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Recombinant Chimeric Yellow Fever-Dengue Type 2 Virus Is Immunogenic and Protective in Nonhuman Primates

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A chimeric yellow fever (YF)-dengue type 2 (dengue-2) virus (ChimeriVax-D2) was constructed using a recombinant cDNA infectious clone of a YF vaccine strain (YF 17D) as a backbone into which we inserted the premembrane (prM) and envelope (E) genes of dengue-2 virus (strain PUO-218 from a case of dengue fever in Bangkok, Thailand). The chimeric virus was recovered from the supernatant of Vero cells transfected with RNA transcripts and amplified once in these cells to yield a titer of 6.3 $\log_{10}$ PFU/ml. The ChimeriVax-D2 was not neurovirulent for 4-week-old outbred mice inoculated intracerebrally. This virus was evaluated in rhesus monkeys for its safety (induction of viremia) and protective efficacy (induction of anti-dengue-2 neutralizing antibodies and protection against challenge). In one experiment, groups of non-YF-immune monkeys received graded doses of ChimeriVax-D2; a control group received only the vaccine diluents. All monkeys (except the control group) developed a brief viremia and showed no signs of illness. Sixty-two days postimmunization, animals were challenged with 5.0 $\log_{10}$ focus forming units (FFU) of a wild-type dengue-2 virus. No viremia (<1.7 $\log_{10}$ FFU/ml) was detected in any vaccinated group, whereas all animals in the placebo control group developed viremia. All vaccinated monkeys developed neutralizing antibodies in a dose-dependent response. In another experiment, viremia and production of neutralizing antibodies were determined in YF-immune monkeys that received either ChimeriVax-D2 or a wild-type dengue-2 virus. Low viremia was detected in ChimeriVax-D2-inoculated monkeys, whereas all dengue-2-immunized animals became viremic. All of these animals were protected against challenge with a wild-type dengue-2 virus, whereas all YF-immune monkeys and nonimmune controls became viremic upon challenge. Genetic stability of ChimeriVax-D2 was assessed by continuous in vitro passage in VeroPM cells. The titer of ChimeriVax-D2, the attenuated phenotype for 4-week-old mice, and the sequence of the inserted prME genes were unchanged after 18 passages in Vero cells. The high replication efficiency, attenuation phenotype in mice and monkeys, immunogenicity and protective efficacy, and genomic stability of ChimeriVax-D2 justify it as a novel vaccine candidate to be evaluated in humans.

Dengue virus is a worldwide public health problem. Over 2 billion people are at risk of dengue virus infections. Annually, 100 million cases of primary dengue fever and over 450,000 cases of dengue hemorrhagic fever (DHF) occur (16). *Dengue virus* is a member of the *Flavivirus* genus within the family *Flaviviridae*, which contains approximately 70 viruses. Sixty-seven of these viruses are transmitted by arthropod vectors, and more than half (38 viruses) have been associated with human diseases. Four serotypes of dengue virus (types 1 through 4) are distinguished by neutralization test (24) and constitute a distinct antigenic complex among the flaviviruses (3). The amino acid homology between the four dengue serotypes is 63 to 68% compared to 44 to 51% between dengue, yellow fever, and West Nile viruses (5, 10, 15). The virus is transmitted to humans by *Aedes aegypti* mosquitoes and causes epidemics involving millions of people inhabiting tropical areas of Asia, Africa, Australia, and the Americas.

Wild-type dengue viruses replicate in the brain tissues of suckling mice and hamsters upon intracerebral (i.c.) inoculation without producing illness (1). However, upon continuous passage in suckling mouse brain, neuroadapted dengue viruses with a low level of virulence for humans were produced (25, 26, 31). Unlike what is found for most flaviviruses, there is no correlation between neurovirulence in mice and visceral virulence in humans for dengue viruses. Currently, the most suitable animal models for dengue virus infections are Old World monkeys, New World monkeys, and apes, which develop subclinical infection and viremia (23, 28, 30). Infected individuals are protected against homotypic infection, probably for life, but cross protection between dengue virus serotypes is short (less than 12 weeks) (25). Therefore, multiple infections with different serotypes are possible. Generally, the most severe illness (DHF) and death occur when individuals become infected with heterologous serotypes due to antibody-dependent enhancement of infection (11, 12). Today it is generally accepted that a tetravalent vaccine is required to induce protective immunity against all four serotypes in order to avoid sensitizing vaccinees to DHF. For the last 50 years many approaches have been undertaken to produce effective dengue vaccines. Although dengue viruses have been satisfactorily attenuated (e.g., PR-159 [S1] for dengue type 2 [dengue-2]) (27), in many cases in vitro or in vivo attenuation was not reproducible in humans. A current strategy is to test selected live virus vaccine candidates stepwise in small numbers of human volunteers. Many laboratories around the world are exploring various strategies to produce suitable vaccine candidates. These range from subunit vaccines (protein vaccine or DNA vaccine), including the prME proteins of dengue viruses, to killed whole-virus vaccines (20), to live attenuated viruses (pro-

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duced by tissue culture passage or recombinant DNA technology) (6, 13). Although some of these candidates have shown promise in preclinical tests and human volunteers, development of a successful dengue vaccine remains to be implemented. In this paper we evaluate a chimeric yellow fever (YF)-dengue-2 virus as a live attenuated vaccine candidate against dengue-2 virus infection. This technology is also applicable to the rapid development of vaccines for other dengue virus serotypes.

MATERIALS AND METHODS

Production of ChimeriVax-D2 virus. The construction of a full-length cDNA used in ChimeriVax-D2 utilizes the two-plasmid system (21) already described in detail for construction of a YF-Japanese encephalitis virus chimera (4). The dengue-2 virus (strain PUO-218) was originally isolated from a child with primary dengue virus infection and classical dengue fever during the 1980 epidemic in Bangkok, Thailand, by D. S. Burke. The virus was isolated by inoculation of plasma into *Toxorhynchites splendens* mosquitoes and amplification in LLC-MK_2 cells (D. S. Burke, unpublished data). Virus was obtained from The Queensland Institute of Medical Research, Brisbane, Australia, and amplified once in C6/36 cells without plaque purification (7). The appropriate fragments of the prME genes of DEN-2 were derived by PCR amplification from the dengue-2 virus clone MON310 (furnished by P. Wright [Department of Microbiology, Monash University, Clayton, Victoria, Australia]). MON310 virus contained the prME genes of the PUO-218 strain in New Guinea C (NGC) virus background (8). The rationale for use of this dengue virus was that this clone was already available in a two-plasmid system similar to ours and that the complete sequence was known, which facilitated its construction with minimum modification.

Plasmids YF5′3′IV/DEN(prME′) and YFM5.2/DEN(E′-E) were digested with *Sph*I and *Aat*II restriction enzymes, YF and dengue virus sequence-encoding fragments were isolated and ligated in vitro using T4 DNA ligase (21). After digestion with *Xho*I to allow runoff transcription, DNA (50 ng of purified template) was transcribed from an SP6 promoter and the integrity of RNA transcripts was verified by nondenaturing agarose gel electrophoresis. Vero cells were transfected with YF-dengue-2 virus RNA using Lipofectin (Gibco/BRL, Life Technologies, Rockville, Md.), and virus was recovered from the supernatants, amplified once in Vero cells, and titrated in a standard plaque assay on Vero cells. The virus (ChimeriVax-D2) titer was 6.3 $\log_{10}$ PFU/ml.

Nucleotide sequencing of ChimeriVax-D2 virus. Vero cells were infected with ChimeriVax-D2 at a multiplicity of infection (MOI) of 0.1. After 96 h, cells were harvested with Trizol (Gibco/BRL, Life Technologies) for RNA isolation. Reverse transcription was performed with Superscript II reverse transcriptase (RT) and a long-RT protocol (Gibco/BRL, Life Technologies), followed by RNaseH treatment (Promega, Madison, Wis.) and long PCR (XL PCR; Perkin-Elmer/ABI, Foster City, Calif.). RT, PCR, and sequencing primers were designed using the YF 17D strain sequence (GenBank accession no. K02749) and the PUO-218 dengue-2 virus strain sequence (GenBank accession no. D00345) as references. For whole-genome sequencing, RT primers used were yf10.8(−), 5′-AGTG GTTTTGTGTTTGTC, and yf5.4(−), 5′-AGTTAACAACCCTAGTTG; PCR primer pairs were yf18(+), 5′-AGTAAATCCTGTGTGCTA, and yf3.5(−), 5′-CAGATGGCTTTCATGCGT, yf3.2(+), 5′-TGCCGAGATCAATCGGAGG CC, and yf5.4(−), and yf5.0(+), 5′-CTTCAGGATCTCCTATTGTTA, and yf10.8(−). For prME region sequencing the RT primer used was yf2.6(−), 5′-AAGAGGCTTTCACTATTGATG, and the PCR primer pair were yf0.2(+), 5′-ATGGTACGACGAGGAGTTCGC, and yf2.6(−). PCR products were gel purified (Qiaquick gel extraction kit; Qiagen) and sequenced using the Dye-Terminator dRhodamine sequencing reaction mixture (Perkin-Elmer/ABI). Sequencing reactions were analyzed on a model 310 genetic analyzer (Perkin-Elmer/ABI), and DNA sequences were evaluated using Sequencher, version 3.0 (GeneCodes), software.

Cells and viruses. Vero and C6/36 cells were obtained from the American Type Culture Collection, Manassas, Va. VeroPM cells (obtained from Aventis Pasteur, Lyon, France) were used at passages between 141 and 151. In addition to ChimeriVax-D2, YF vaccine (YF-Vax [Aventis Pasteur] and ArilVax [Medeva Pharma Ltd., Leatherhead, Surrey, United Kingdom]) viruses and dengue-2 virus (S16803; provided by Kenneth Eckels, Walter Reed Army Medical Research Institute, Forest Glen, Md.) were used in this study.

Animal studies. (i) Mice. For studies of neurovirulence, 4-week-old outbred (ICR) mice (Taconic Farms, Inc., Germantown, N.Y.) were inoculated by the i.c. route with 0.03 ml of virus inocula or phosphate-buffered saline (PBS). Suckling mice (3 to 9 days old) were born on site from pregnant ICR (CD-1) mice (Charles River Laboratories, Wilmington, Mass.) and inoculated by the i.c. or intraperitoneal (i.p.) route with 0.02 ml. Animals were observed for 21 days, and deaths were recorded. Moribund animals were euthanized under anesthesia.

For immunogenicity studies, 4-week-old ICR mice were immunized subcutaneously (s.c.) with 0.1 ml of virus and bled 4 weeks later for determination of neutralizing antibodies in sera.

(ii) Monkeys. Two experiments were performed at the Tulane Regional Primate Research Center (Covington, La.) with healthy young-adult, colony-reared Indian rhesus monkeys (*Macacca mulatta*). Studies were carried out under an approved protocol 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* (19a).

(a) Experiment 1. Dose-response studies were performed with 22 male monkeys weighing 2.1 to 3.1 kg. All animals were previously determined to be negative for antibodies against YF, dengue, and St. Louis encephalitis viruses by hemagglutination inhibition test (performed by Robert Shope, University of Texas, Medical Branch, Galveston, Tex.). Animals were randomly divided into five groups and immunized s.c. with 5.0, 4.0, 3.0, or 2.0 $\log_{10}$ PFU of ChimeriVax-D2 or were sham inoculated with PBS in the left arm. Vaccine aliquots were frozen for back titration. Blood was collected from the femoral vein under anesthesia immediately before vaccination, then daily for 8 days to determine viremia, and on days 15 and 30 for assessment of neutralizing antibodies. Animals were clinically evaluated for signs of illness. Blood was drawn on day 63, and the monkeys were challenged s.c. with 5 $\log_{10}$ focus-forming units (FFU; see below) of dengue-2 (S16803) virus. For the following 8 days, blood was collected for determination of viremia and animals were observed for signs of illness. Two weeks postchallenge animals were bled for serology and released from the study.

(b) Experiment 2. The objective of this experiment was to determine whether preimmunity to YF 17D would interfere with immunization by ChimeriVax-D2 virus. Fourteen male monkeys (with or without preimmunity to YF 17D vaccine) were divided into four groups. Twelve monkeys that had been immunized with ArilVax YF vaccine 4 months previously were inoculated s.c. with ChimeriVax-D2, wild-type dengue-2 virus, ArilVax YF vaccine, or PBS. Two non-YF-immune controls received PBS. Blood was drawn daily for the next 9 days (for assessment of viremia) and at 14, 28, 47, and 118 days (for titration of neutralizing antibodies). All monkeys were challenged (as described for experiment 1) on day 118. For the following 9 days, monkeys were clinically evaluated and bled for assessment of viremia. Twenty-five days postchallenge, animals were bled for determination of neutralizing antibodies and then released to their colonies.

Determination of viremia in monkey sera. The concentration of ChimeriVax-D2 virus in serum samples was determined by plaque assay of Vero cell monolayers. Undiluted serum or serial 10-fold dilutions of serum (in duplicate) were inoculated onto Vero cells grown in minimal essential medium (MEM)–5% fetal bovine serum (FBS) in 12-well culture plates. After 1 h of adsorption at 37°C, wells were overlaid with 1 ml of MEM supplemented with 10% FBS, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 0.75% methylcellulose. Plates were incubated for 5 days at 37°C in 5% CO_2. Monolayers were fixed by addition of 1 ml of 20% formalin solution to the overlay medium. After 1 h or greater of fixation at room temperature, the fixative was removed, wells were washed with water, and monolayers were stained with 1% crystal violet in 70% methanol. Plaques were counted, and titers were expressed as PFU per milliliter.

The concentration of wild-type dengue-2 virus (challenge strain S16803) in serum samples was determined by an immunocytochemical focus-forming assay using C6/36 mosquito cells. We developed this technique because dengue-2 virus did not produce distinct plaques in Vero cells. Serial 10-fold dilutions of serum (in duplicate) were inoculated onto monolayers of C6/36 cells (grown in MEM–10% FBS–1% nonessential amino acids) and incubated for 1 h at 28°C for adsorption. Wells were overlaid as described above and incubated for 6 days at 28°C. Cell monolayers were fixed for 1 h or greater by addition of 1 ml of 20% formalin. Wells were washed with PBS containing 0.05% Tween 20, and nonspecific binding sites were blocked with PBS containing 0.05% Tween 20 and 2.5% nonfat dry milk (blocking buffer). Wells were treated sequentially with a monoclonal antibody against dengue-2 virus (Chemicon International, Inc., Temecula, Calif.) and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Southern Biotechnology Associates, Birmingham, Ala.), each diluted 1:500 in blocking buffer. Antibody-bound foci of infection were developed with the insoluble alkaline phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Sigma Chemical Co., St. Louis, Mo.) and counted, and titers were expressed as FFU per milliliter.

Plaque reduction neutralization test. For determination of dengue virus-neutralizing antibody titers, serial twofold dilutions of serum (starting at a serum dilution of 1:5) were mixed with equal volumes of a suspension of 1,000 FFU of dengue-2 virus/ml. The mixtures contained 5% fresh-frozen rabbit serum (Accurate Chemical, Westbury, N.Y.) as a source of complement. The serum-virus mixtures were incubated overnight at 4°C and tested (0.1 ml/well) for concentration of infectious virus using the focus-forming assay described above. The neutralization titer was defined as the lowest serum dilution at which the infectious virus concentration was reduced by 50% from the concentration found when virus was incubated with culture medium rather than serum.

RESULTS

Nucleotide sequencing of YF-dengue-2 virus chimera. In Tables 1 and 2 the amino acid sequence in the prME region of ChimeriVax-D2 is compared with published sequences for PUO-218 (7), prototype NGC virus (7), and an attenuated dengue-2 virus vaccine, strain PR-159 (S1) (10). The amino acid sequence of the ChimeriVax-D2 virus prME differed from

TABLE 1. Amino acid sequence comparison of prM protein of dengue-2 viruses

Virus	Amino acid residue in prM at position:						
	28	31	55	57	125	152	161
ChimeriVax-D2	E	V	L	R	I	A	V
PUO-218	E	V	L	R	I	A	V
NGC	E	V	F	R	T	A	V
PR-159 (S1)	K	T	F	K	T	V	I

that of parental PUO-218 at position 484. This residue, which is a part of the envelope transmembrane region, is amino acid I in PUO-218 and PR-159 (S1) and is V in ChimeriVax-D2 and NGC virus. In addition, a *Nar*I site was intentionally introduced at the 3′ end of the E (E-NS1 junction) coding sequence, resulting in amino acid change Q494G (residues at this position are not compared in Table 2). The PUO-218 virus differs from NGC virus in two amino acids in prM (residues 55 and 125, respectively L and I in PUO-218 instead of F and T in NGC) and six amino acids in the E protein (amino acid 71 is E in PUO-218 and D in NGC [71 E→D], 126 E→K, 141 V→I, 164 V→I, 402 F→I and 484 I→V) (Table 2). In Table 3 nucleotide and amino acid differences within the nonstructural (NS) genes of ChimeriVax-D2, ChimeriVax-JE (9), and the parent YF 17D virus (22) are shown. There were six nucleotide differences between ChimeriVax-D2 and YF 17D. Four substitutions at nucleotide positions 5641, 6898, 8212, and 10454 are silent and do not cause amino acid changes. Substitutions at positions 4025 and 7319 result in amino acid changes within NS2A (V104M) and NS4B (E7K), respectively. These are the same amino acid changes previously reported for ChimeriVax-JE virus (9). Within the YF 17D genes, there were three nucleotide differences between ChimeriVax-JE and ChimeriVax-D2 (nucleotide positions 5461, 6898, and 8581). These changes were silent and did not result in amino acid substitutions (Table 3).

Growth kinetics in cell cultures. The growth kinetics of the ChimeriVax-D2 virus was determined in VeroPM cells (a certified cell bank intended for manufacturing all ChimeriVax viruses). Cells were grown (in MEM-alpha–L-glutamine supplemented with 10% FBS) to confluency in a T-75 tissue culture flask and inoculated with the virus at an MOI of 0.025. After 1 h of incubation at 37°C, medium containing 3% FBS was added and the flask was returned to a CO_2 incubator. Every 24 h 0.5 ml of cell culture supernatant was removed, FBS was added to a final concentration of 20%, and the samples were frozen for infectivity titration by plaque assay. The virus titers for days 1 to 4 were 6.39, 7.82, 7.76, and 6.93 $\log_{10}$ PFU/ml, respectively. The peak titer was on day 2, coincident with the appearance of cytopathic effect (CPE); titers decreased on the following days as CPE progressed. Reduction in infectious virus titer may have been due to the death of host cells and virus degradation in supernatant fluid at the 37°C temperature.

Neurovirulence phenotype in suckling mice. Although mouse neurovirulence does not predict virulence or attenuation of dengue viruses for humans, it was important to demonstrate that the ChimeriVax-D2 virus does not exceed its parent YF 17D virus in neuroinvasiveness and neurovirulence. The YF 17D vaccine virus retains a degree of neurotropism for mice (kills mice of all ages after i.c. inoculation) and causes (generally subclinical) encephalitis in monkeys after i.c. inoculation (17). In initial studies, groups of 4-week-old mice were inoculated by the i.c. route with various doses of either ChimeriVax-D2 or YF-Vax as shown in Table 4 and observed for paralysis or death for 21 days. Seven of 8, 6 of 8, 3 of 8, and 2 of 8 mice that received 3.0, 2.0, 1.0, or 0.1 $\log_{10}$ PFU of YF-Vax, respectively, died. The average survival time (AST) was dose dependent, ranging from 8.7 (for 3 $\log_{10}$ PFU) to 12 days (for 0.1 $\log_{10}$ PFU). In contrast, all mice that received ChimeriVax-D2 (even at 3 log units higher than the highest YF-Vax dose) survived the i.c. challenge.

To determine the age at which mice become resistant to i.c. challenge with ChimeriVax-D2, groups of 3- to 9-day-old suckling mice were inoculated with 4 $\log_{10}$ PFU of ChimeriVax-D2 and observed for paralysis or death for 21 days. For controls, similar age groups were sham-inoculated i.c. with PBS or with 3 $\log_{10}$ PFU of unpassaged commercial YF vaccine (YF-Vax) by the i.p. route (it was not necessary to inoculate suckling mice with YF-Vax by the i.c. route because we had consistently shown that this vaccine was virulent for suckling or 4-week-old mice by this route) (9).

All suckling mice (3 to 7 days old) inoculated by the i.c. route with the ChimeriVax-D2 virus died between 10 and 14 days postinoculation, whereas 8 out of 10 suckling mice (9 days old) survived. The AST was 9.9 days for 3-day-old mice and increased to 14.5 days for 9-day-old mice. Similarly, all suckling mice (3 to 5 days old) inoculated with YF-Vax by the i.p. route, using a dose 10-fold lower than that for the ChimeriVax-D2 virus, died between 10 and 13 days after inoculation. Nine out of 10 9-day-old mice as well as 7 of 10 7-day-old mice inoculated with YF-Vax survived. All sham-immunized suckling mice survived the 21-day observation period. Results similar to those obtained with ChimeriVax-D2 had been obtained previously with suckling mice inoculated with ChimeriVax-JE virus (9).

Viremia, immunogenicity, and protection against challenge in nonhuman primates. The wild-type dengue-2 virus (strain S16803) has been shown to induce a high level of viremia (reaching 5.0 $\log_{10}$ PFU) in rhesus monkeys, which lasts for a mean duration of 7 days (20). Attenuation of dengue-2 virus vaccine candidates can therefore be estimated by comparing the level and duration of viremia with those for reference wild-type strains. To define the safety (viremia profile) and efficacy (neutralizing antibody responses and protection against challenge with wild-type dengue-2 virus) of the ChimeriVax-D2 vaccine candidate, two experiments were carried out.

TABLE 2. Amino acid sequence comparison of E protein of dengue-2 viruses

Virus	Amino acid residue in E at position:														
	71	81	126	129	139	141	162	164	202	203	335	352	390	402	484
ChimeriVax-D2	E	S	E	V	I	V	I	V	E	N	I	I	N	F	V
PUO-218	E	S	E	V	I	V	I	V	E	N	I	I	N	F	I
NGC	D	S	K	V	I	I	I	I	E	N	I	I	N	I	V
PR-159 (S1)	D	T	E	I	V	I	V	I	K	D	T	T	D	F	I

TABLE 3. Nucleotide sequence differences between NS genes of ChimeriVax viruses and YF 17D vaccine virus

Gene	NT position[a]	NT of:			AA substitution[d]
		ChimeriVax-		YF 17D[e]	
		JE[b]	D2[c]		
NS2A	4025	A	A	G	M/V
NS3	5461	C	T	T	
	5641	G	G	A	
NS4A	6898	C	A	C	
NS4B	7319	A	A	G	K/E
NS5	8212	T	T	C	
	8581	C	A	A	
3′ NC	10454	A	A	G	

[a] Nucleotide (NT) positions are numbered from the 5′ terminus of the YF 17D genome.
[b] ChimeriVax is the chimeric YF-JE research virus resulting from two Vero cell passages and one FeRhL cell passage posttransfection (9).
[c] ChimeriVax-D2 resulted from three Vero cell passages posttransfection.
[d] ChimeriVax amino acid (AA)/YF 17D AA.
[e] From reference 22.

(i) Experiment 1: dose-response effectiveness of ChimeriVax-D2 in monkeys. The goal of this experiment was to determine the minimum vaccine dose required for protection against challenge with wild-type dengue-2 virus. It was anticipated that this experiment would also define the viremia profile of the ChimeriVax-D2 virus in non-YF-immune monkeys and would determine if immunization with a single dose results in protection of animals against challenge virus. Protection was defined as reduction of viremia in test monkeys compared to that in the PBS control group.

As shown in Table 5, all monkeys became viremic. There was no significant difference in the magnitude of viremia between the high- and low-dose groups, with mean peak titers ranging from 1.34 to 1.65 $\log_{10}$ PFU. However, the mean duration of viremia in the high-dose group (5 $\log_{10}$ PFU) was 4.25 $\pm$ 1.7 days, which was 1 day longer than that for the low-dose group (2 $\log_{10}$ PFU; 3.25 $\pm$ 1.3 days) ($P = 0.46$, which was not significant according to the Wilcoxon rank sum test). Animals were bled on days 15 and 30 and immediately before challenge (day 60), and neutralizing antibody titers in serum were measured in a plaque reduction neutralization test against the heterologous virus (dengue-2 virus strain S16803) (Table 6).

All monkeys developed anti-dengue-2 virus neutralizing antibodies on day 15 except monkey AI25. This monkey, which was in the lowest-dose group, developed a neutralizing titer of 640 on day 30. Lower doses of the vaccine resulted in lower geometric mean titers (GMT) (the GMT was 640 for the high-dose group and dropped to 32 for the lowest-dose group). However, by day 30 postimmunization, all monkeys developed high titers of neutralizing antibodies and GMT did not differ across groups. Upon challenge, no viremia was detected in any immunized monkey, whereas all four unimmunized controls became viremic with a mean peak titer of 3.6 $\log_{10}$ FFU and a mean duration of 5 days (not shown). In all animals except one (1H02), strong anamnestic responses were observed after challenge (Table 6). This experiment demonstrated that, even at its lowest dose (2 $\log_{10}$ PFU), ChimeriVax-D2 had sufficiently replicated in its host and protected animals from infection by the challenge virus.

(ii) Experiment 2: determination of viremia, immunogenicity, and protection of ChimeriVax-D2 in YF-preimmune monkeys. In Table 7, the viremia profile of YF-immune monkeys that received ChimeriVax-D2 is compared to that of similar monkeys that received wild-type dengue-2 virus. Low viremia (0.7 $\log_{10}$ PFU/ml) was detected in monkeys immunized with ChimeriVax-D2, whereas all monkeys that received the wild-type dengue-2 virus developed viremia lasting 4 to 5 days, with peak titers between 4.7 and 5.5 $\log_{10}$ FFU/ml.

To determine whether the chimeric virus sufficiently replicated in monkeys, animals were bled on days 28 and 118 (prior to challenge) and sera were tested for neutralizing antibodies to wild-type dengue-2 virus (S16803). Animals were then challenged with 5.0 $\log_{10}$ FFU of wild-type dengue-2 virus (S16803). Viremia was measured from day 1 to day 9 postchallenge in an immunocytochemical focus-forming assay using C6/36 cells (Table 8). All YF-immune monkeys developed neutralizing antibodies against dengue-2 virus after immunization with either ChimeriVax-D2 or dengue-2 virus (Table 8). However, the levels of antibody were higher in monkeys infected with wild-type dengue-2 virus. Upon challenge with dengue-2 virus, none of the monkeys in the ChimeriVax-D2 or the dengue-2 virus group developed viremia, whereas all YF-immune and nonimmune monkeys developed high levels of viremia. This indicated that, despite the low viremia, monkeys immunized s.c. with ChimeriVax-D2 were protected against severe challenge with wild-type dengue-2 virus. There was no cross protection between YF and dengue-2 virus, since none of the YF-immune monkeys were protected against dengue-2 virus challenge, as shown by the high level of viremia in these animals, which was similar to that in control group monkeys. The mean peak of viremia in unprotected animals was 3.5 to 4.7 $\log_{10}$ FFU/ml, and the mean duration was 4 to 5 days (Table 9).

Assessment of genomic stability and neurovirulence of in vitro-passaged ChimeriVax-D2 virus: Stability of prME genes of ChimeriVax-D2 virus in vitro. We conducted an experiment to determine the stability of the dengue virus prME and YF genes during sequential passage in vitro. The ChimeriVax-D2 virus at passage 2 posttransfection was used to inoculate a 25-cm^2 flask of Vero cells. Total RNA was isolated, and the

TABLE 4. Neurovirulence and neuroinvasiveness of ChimeriVax-D2 and YF 17D viruses in outbred ICR mice

Virus	Dose[a]	Age (days)	Route	No. dead/ no. inoculated (% mortality rate)	AST (days)
ChimeriVax-D2	6.0	28	i.c.	0/5 (0)	
	5.0	28	i.c.	0/5 (0)	
	4.0	28	i.c.	0/5 (0)	
	4.0	9	i.c.	2/10 (20)	14.50
	4.0	7	i.c.	10/10 (100)	10.20
	4.0	5	i.c.	11/11 (100)	10.18
	4.0	3	i.c.	14/14 (100)	9.93
YF 17D[b]	3.0	28	i.c.	7/8 (87.5)	8.7
	2.0	28	i.c.	6/8 (75)	9.3
	1.0	28	i.c.	3/8 (37)	12.3
	0.1	28	i.c.	2/8 (25)	12.00
	3.0	9	i.p.	1/10 (10)	12.00
	3.0	7	i.p.	2/10 (20)	10.50
	3.0	5	i.p.	11/11 (100)	11.00
	3.0	3	i.p.	9/9 (100)	9.00
None (PBS only)		3	i.c.	0/12 (0)	
		5	i.c.	0/9 (0)	
		7	i.c.	0/6 (0)	
		9	i.c.	0/8 (0)	

[a] Doses of inocula are expressed as $\log_{10}$ PFU and were confirmed by back titration of samples in a plaque assay.
[b] YF-Vax was rehydrated with diluents supplied by the manufacturer and used without further passage.

TABLE 5. Viremia in rhesus monkeys immunized with graded doses of ChimeriVax-D2 virus

Monkey	ChimeriVax-D2 dose ($\log_{10}$ PFU)	Viremia ($\log_{10}$ PFU/ml) by postimmunization day:								Mean	
		1	2	3	4	5	6	7	8	Peak titer[b] (SD)	Duration[c] (SD)
AG88	5	0[a]	1.0	0	1.0	0	0	0	0	1.40 (0.3)	4.25 (1.7)
AK37		0	0.7	0	1.0	1.0	0	1.5	1.0		
1H02		0	1.5	0.7	0.7	0.7	0	0	0		
AK17		0	0	1.3	0.7	0.7	1.6	0.7	1.3		
AH01	4	0	0	0	1.2	1.0	0	0	0	1.34 (0.4)	4.0 (2)
AI68		1.3	1.4	1.5	1.5	1.3	0	0.7	1.4		
AJ41		0	0	0	0.7	0	0	0.7	0		
AK97		0	1.8	1.8	1.0	0.7	0	0	0		
AP31		0	1.2	1.2	0.7	1.4	1.5	0	0		
AP02	3	0	0	0	1.2	1.8	1.8	0.7	0	1.40 (0.4)	4.0 (1.9)
AG36		0	0.7	1.6	1.7	1.7	1.5	0	0.7		
AP08		0.7	0	1.0	0.7	1.3	0	0	0		
AN98		0	0	0.7	1.5	1.5	0.7	0.7	0		
AP09		0	0	0	0	0	0.7	0	0		
AG14	2	0	0	0	1.0	2.4	1.9	0	0	1.65 (0.6)	3.5 (1.3)
AI25		0	0	0	1.4	1.7	0	0	0		
AH63		0	0	1.5	1.5	1.4	1.5	1.0	0		
AK92		0	0	0.7	0.7	1.0	0.7	0	0		
AH90	None (PBS)	NT[d]	NT	NT	NT	NT	NT	NT	NT		
AP50		NT	NT	NT	NT	NT	NT	NT	NT		
AK95		NT	NT	NT	NT	NT	NT	NT	NT		
AP05		NT	NT	NT	NT	NT	NT	NT	NT		

[a] <0.7 $\log_{10}$ PFU/ml.
[b] Log_{10} PFU/ml.
[c] Days.
[d] NT, not tested.

complete nucleotide sequence of the virus was determined (passage 3) and compared to the published sequence of dengue-2 virus strains PUO-218 (7) and YF 17D (22) (Tables 1 to 3). There were five nucleotide differences found (nucleotide 2429 was A in PUO-218 and G in ChimeriVax-D2 [2429 A→G], 2431 T→C, 2434 A→G, 2437 A→G, and 2452 C→T) when the envelope sequence of ChimeriVax-D2 was compared to its parent dengue-2 (PUO-218 strain) virus. Substitution at positions 2429 and 2431 resulted in an amino acid change at position 484 from I to V (Table 2), whereas substitutions at positions 2434, 2437, and 2452 were silent and did not result in any amino acid changes. Within the YF genes, the nucleotide sequences of ChimeriVax-D2 differed from the nucleotide sequences of YF 17D (22) at six positions (described above in the discussion of nucleotide sequencing) (Table 3). To determine if the chimeric virus was genetically stable upon in vitro passage, the passage 3 virus was continuously passaged in VeroPM cells (passages 141 to 147) at an MOI of 0.1 to 0.5 up to 18 times. Viruses harvested at passages 3, 5, 8, 10, 13, 16, and 18 were titrated, sequenced (passages 3, 5, 10, and 15), and inoculated (passages 3, 5, 10, and 18) into groups of 4-week-old mice ($n = 5$) by the i.c. route to assess the neurovirulence phenotype. The titer of virus (6.6 to 7.8 $\log_{10}$ PFU/ml) at each passage, as well as plaque morphology (large plaques), remained unchanged, indicating that there were no plaque variants overgrowing the original parent population. No additional mutations were found in the prME genes of the chimera (compared to passage 3 virus) upon 18 passages in VeroPM cells. Within the YF genes, however, the passage 18 virus appeared to be heterogeneous at position 3524 (both parent nucleotide G and mutant nucleotide A were present). This would translate into a mixture of E and K amino acids at position 354 of the NS1 protein. Similar to passage 3 virus, passage 5, 10, 13, 16, and 18 viruses were avirulent for 4-week-old mice inoculated by the i.c. route (5 $\log_{10}$ PFU was the highest dose tested) (Table 10). There were no significant differences in production of anti-dengue-2 virus neutralizing antibodies (sera were collected 38 days postinoculation) across 18 passages, demonstrating that the immunogenicity of ChimeriVax-D2 is not altered upon passage in Vero cells. The GMT of neutralizing antibodies were 61, 46, 46, and 53 for animals that received 5 $\log_{10}$ PFU of passage 3, 5, 10, and 18 virus, respectively.

DISCUSSION

The successful strategy previously described for construction of ChimeriVax-JE (4) was employed to generate a chimeric YF-dengue-2 virus (ChimeriVax-D2). The virus was replication competent and grew to 7.8 $\log_{10}$ PFU/ml after 2 days of culture in VeroPM cells inoculated at an MOI of 0.025.

The envelope genes of the ChimeriVax-D2 virus were derived from the PUO-218 strain of dengue-2 virus, originally isolated from a 21-month-old child with a symptomatic primary dengue-2 virus infection during the 1980 epidemic in Bangkok. The virus was originally isolated by inoculation of plasma into *T. splendens* mosquitoes and passaged once in LLC-MK_2 cells and once in C6/36 cells before cloning (7). The PUO-218 virus is closely related to the NGC virus strain on the basis of nucleotide sequence comparison (7). Within the products of the envelope genes, it differs from NGC virus in two amino acid residues in prM (55 F→L and 125 T→I) and six amino acids in the E protein (71D→E, 126K→E, 141I→V, 164I→V, 402I→F, and 484V→I). Residue 126 of the E protein, which is the basic amino acid K in NGC virus and the acidic amino acid

TABLE 6. Neutralizing antibody titers[a] in rhesus monkeys immunized with graded doses of ChimeriVax-D2

Monkey	ChimeriVax-D2 dose ($\log_{10}$ PFU)	Titer on postimmunization day:			Titer on postchallenge day 14
		0[c]	15	30	
AG88	5	0[d]	1,280	320	20,480
AK37		0	640	640	1,280
1H02		0	320	640	160
AK17		0	640	160	640
GMT[b] (SD)			640 (402)	380 (240)	1,280 (9,904)
AH01	4	0	160	640	2,560
AI68		0	1,280	320	1,280
AJ41		0	160	320	1,280
AK97		0	320	640	20,480
AP31		0	160	80	10,240
GMT (SD)			279 (488)	320 (240)	3,880 (8,325)
AP02	3	0	320	320	20,480
AG36		0	320	640	1,280
AP08		0	80	640	40,960
AN98		0	160	80	640
AP09		0	40	160	640
GMT (SD)			139 (131)	279 (263)	3,378 (17,891)
AG14	2	0	40	320	81,920
AI25		0	0	640	1,280
AH63		0	20	320	5,120
AK92		20	1,280	160	1,280
GMT (SD)		20 (10)	32 (630)	320 (201)	5,120 (39,721)
AH90	None (PBS)	0	0	0	1,280
AP50		0	0	0	2,560
AK95		0	0	0	2,560
AP05		0	10	0	320
GMT (SD)			10 (5)		1,280 (1,089)

[a] 50% plaque reduction neutralization was measured against heterologous virus (challenge virus). Monkeys were challenged by the s.c. route with 5.0 $\log_{10}$ FFU of wild-type dengue-2 viruses on day 63 postimmunization.
[b] GMT were measured from the last dilution of sera, which resulted in 50% reduction in number of virus plaques.
[c] Preimmunization sera.
[d] Titers <10.

E in PUO-218 or PR-159 (S1 vaccine strain), is believed to be required for the neurovirulence phenotype of the mouse-adapted NGC virus (2, 8). A similar change in amino acid charge (K to E) was found at position 138 on the E protein of JE virus, which was shown to be responsible for its mouse neurovirulence (29).

Although wild-type unpassaged dengue viruses replicate in brains of suckling mice and hamsters inoculated by the i.c. route (1), they usually induce subclinical infections, and death occurs only in rare cases. However, neurovirulence for mice can be achieved by adaptation through sequential passage in mouse brain. Such neuroadapted viruses may be attenuated for humans. For example, NGC virus, the prototype dengue-2 virus isolated in 1944 and introduced into the Americas in 1981, is not neurovirulent for suckling mice; however, after sequential passage in mouse brain it became neurovirulent for mice but was attenuated for humans (25, 26, 31). When the prME genes of the PUO-218 strain (nonneurovirulent for mice) were inserted into the mouse neuroadapted NGC virus backbone, the chimeric virus (MON310) was attenuated for 3-day-old BALB/c mice inoculated i.c. (8), confirming the previous observation (2) that the mouse neurovirulence of the neuroadapted NGC virus resides solely in envelope protein E. Similarly, when the envelope genes of YF 17D were replaced with those of the PUO-218 virus, the resulting ChimeriVax-D2 virus was attenuated for mice, in contrast to YF 17D parent virus, which killed mice with a 50% lethal dose of ≤100 PFU (Table 4) (9). Mice became resistant to lethal ChimeriVax-D2 infection by the i.c. route at 9 days of age, consistent with our previous observations with ChimeriVax-JE virus (9). As mentioned above, attenuation of dengue-2 viruses for mice may not correlate with attenuation in humans, but it was important to demonstrate that the use of the YF 17D backbone did not increase the neurovirulence of the chimeric virus to the level of YF-Vax. Because the mouse neurovirulence of ChimeriVax-JE correlated well with its neurovirulence for monkeys (9, 19), we anticipated that the ChimeriVax-D2 virus would be less neurovirulent in monkeys than YF 17D.

Attenuation of ChimeriVax-D2 virus was further evaluated by determining its viremia profile in rhesus monkeys, the most suitable preclinical model for assessing virulence of dengue virus infections for humans. Wild-type dengue-2 viruses usually produce 4 to 5 days of viremia in monkeys, with peak virus concentrations of 4 to 5 $\log_{10}$ PFU/ml. Animals recover from infection and acquire immunity to the homologous virus, probably for life. Attenuation of dengue virus vaccine candidates in monkeys can be assessed by comparing the magnitude of viremia after s.c. inoculation with vaccine candidates to that after inoculation with reference wild-type virus. Two experiments were carried out. In the first experiment, monkeys were immunized with graded doses of ChimeriVax-D2 and challenged 63 days later with 5.0 $\log_{10}$ FFU of a wild-type dengue-2 virus. Following immunizations, all monkeys developed low levels of

TABLE 7. Viremia in YF-immune rhesus monkeys inoculated with ChimeriVax-D2 or dengue-2 wild-type virus by the s.c. route

Monkey[a]	Virus (dose)	Viremia ($\log_{10}$ PFU/ml) by postimmunization day:									Mean	
		1	2	3	4	5	6	7	8	9	Peak titer[d] (SD)	Duration[e] (SD)
P351	ChimeriVax-D2 (4 $\log_{10}$ PFU)	0.7	0	0	0	0	0	0.7	0	0	0.7 (0)	2 (1)
P353		0[b]	0	0	0	0	0	0.7	0	0		
P741		0.7	0	0	0	0	0	0.7	0.7	0		
P304	Dengue-2 (4 $\log_{10}$ FFU)	0[c]	0	0	2.6	4.0	4.5	4.8	1.7	0	4.9 (0.4)	4.5 (0.6)
P069		0	0	1.7	2.9	4.7	4.6	2.9	0	0		
P306		0	0	0	3.2	4.7	4.6	3.1	0	0		
P098		0	0	0	0	4.5	5.5	5.4	3.2	0		

[a] Four months prior to this experiment, monkeys were immunized with YF 17D vaccine, and all developed neutralizing antibodies to YF virus at levels ≥1:100.
[b] <0.7 $\log_{10}$ PFU/ml.
[c] <1.7 $\log_{10}$ FFU/ml.
[d] Log_{10} FFU/ml.
[e] Days.

TABLE 8. Viremia (postchallenge), and neutralizing antibody titers (prechallenge) in monkeys inoculated with vaccine viruses and challenged with 5 log_{10} FFU of dengue-2 virus by the s.c. route

Monkey	Vaccine[a]		Prechallenge N titers[b] on day:		Viremia (log_{10} FFU/ml) postchallenge by postimmunization day:						
	1st	2nd	28	118	3[c]	4	5	6	7	8	9
P351	YF 17D	ChimeriVax-D2 (4 log_{10} PFU)	320	NT[e]	0[d]	0	0	0	0	0	0
P353			10	20	0	0	0	0	0	0	0
P741			160	80	0	0	0	0	0	0	0
P304	YF 17D	Dengue-2 virus (4 log_{10} FFU)	2,560	1,280	0	0	0	0	0	0	0
P069			2,560	1,280	0	0	0	0	0	0	0
P306			5,120	2,560	0	0	0	0	0	0	0
P098			5,120	2,560	0	0	0	0	0	0	0
P451	YF 17D	YF 17D (4 log_{10} PFU)	<10	<10	0	0	3.3	4.4	3.7	2.0	0
P258			<10	<10	0	0	4.6	4.8	0	0	0
P846			<10	<10	0	0	2.2	3.4	5.0	3.9	2.3
P796	YF 17D	None	NT	NT	0	2.2	3.8	4.5	5.0	3.2	0
P318			NT	NT	0	2.0	3.5	4.2	4.2	3.2	0
P209	None	None	NT	NT	1.7	2.1	4.0	4.4	4.4	0	0
P719			NT	NT	0	2.5	2.2	2.7	2.5	2.3	0

[a] ArilVax YF vaccine was administered 4 months prior to the second vaccine.
[b] Titers are the highest serum dilutions which resulted in a 50% reduction in number of foci produced by dengue-2 (S16803) virus infection. N, neutralizing antibody.
[c] No viremia was detected prior to this day.
[d] <1.7 log_{10} PFU/ml.
[e] NT, not tested.

viremia. The mean peak titers (1.3 to 1.6 log_{10} PFU) and duration (3.5 to 4.2 days) of viremia were independent of the vaccine dose, indicating that the chimera, even at its lowest dose (2 log_{10} PFU), replicated sufficiently in these animals (Table 5). The onset of viremia was significantly delayed ($P = 0.0472$) in monkeys in the low-dose group compared to the high-dose group. Similarly, inoculation of mice, monkeys, or humans with large doses of YF 17D virus resulted in an earlier appearance of viremia, but viremia was inconsistent, lower in magnitude, and briefer in duration than after inoculation of diluted virus (17). Generally this "prozone effect" is followed by a lower immune response at higher doses of YF 17D virus. This was not the case for ChimeriVax-D2 virus because higher doses of this virus resulted in higher neutralizing antibody responses 2 weeks after immunization (GMT of neutralizing antibodies were 640 and 32 for monkeys that received 5 and 2 log_{10} PFU of virus, respectively) (Table 6). It is possible that YF 17D vaccines, which are produced in eggs (ChimeriVax-D2 was produced in cell culture), contain interferon, defective interfering particles, or noninfectious particles competing for cell receptors.

On day 30, all animals had high titers of neutralizing antibodies, with no differences across the dose groups (the GMT were 380 for the highest-dose group and 320 for the lowest-dose group). An anamnestic response was observed when animals were challenged with dengue-2 virus, with an increase in GMT of about 10-fold across the groups. After challenge, GMT in the immunized groups were higher (up to fourfold) than those of sham-immunized monkeys. No viremia was detected in any immunized animals, whereas all nonimmunized monkeys became viremic (mean peak titer of 3.6 ± 0.3 log_{10} FFU/ml and mean duration of 5.25 ± 0.5 days; data not shown). These experiments demonstrated that the chimeric virus had a low-viremia profile and was immunogenic and protective at the lowest dose (2 log_{10} PFU) inoculated.

In the second experiment animals that had previously (4 months prior to this experiment) been immunized with a YF 17D vaccine received either ChimeriVax-D2 or wild-type dengue-2 (strain S16803) virus. Low viremia (peak titer of 0.7 log_{10} PFU/ml and mean duration of 2 ± 1 days) was detected in ChimeriVax-D2-immunized animals, whereas dengue-2 wild-type virus induced viremia in all monkeys, with a mean peak titer of 4.9 ± 0.4 log_{10} FFU/ml and a mean duration of 4.5 ± 0.6 days (Table 7). The level of viremia (0.7 log_{10} PFU/ml) in YF-immune monkeys was slightly lower than that observed in non-YF-immune animals (mean peak titer, 1.3 to 1.6 log_{10} PFU/ml) (Table 5), in monkeys immunized with 5 log_{10} PFU of YF 17D vaccine (mean peak titer of 1.5 log_{10} PFU/ml; data not shown), and in monkeys that received graded doses (2 to 5 log_{10} PFU) of ChimeriVax-JE (mean peak titer, 1.0 to 2.0 log_{10}

TABLE 9. Immunization and challenge of YF-immune monkeys

1st vaccine	2nd vaccine	Seroconversion[c]		Viremia after dengue-2 virus challenge[d]	Mean	
		Dengue-2	YF		Peak titer[a] (SD)	Duration[b] (SD)
YF 17D	ChimeriVax-D2	3/3	3/3	0/3	<1.7	0
YF 17D	Dengue-2 virus	4/4	4/4	0/4	<1.7	0
YF 17D	YF 17D	0/3	3/3	3/3	4.73 (0.3)	4 (2)
YF 17D	None	0/2	2/2	2/2	4.60 (0.6)	5 (0)
None	None	0/2	0/2	2/2	3.55 (1.2)	5 (0)

[a] Log_{10} FFU/ml.
[b] Days.
[c] Number of monkeys that seroconverted/number of monkeys tested.
[d] Number of monkeys with viremia/number of monkeys tested.

TABLE 10. Genetic stability of ChimeriVax-D2 passed in VeroPM cells

Passage level[a]	Titer[b]	Dose inoculated[c]	Neurovirulence in mice[d]	Amino acid changes[j] in:		GMT[h] ± SD
				D2-PrME[e]	YF[f]	
3	6.6	5	No	E, I484V[g]; E, Q494G[g]	NS2A, V104M[g]; NS4B, E7K[g]	61 ± 477
		4	No			7 ± 15
5	7.2	5	No	No changes	ND	46 ± 16
		4	No			9 ± 20
8	7.3	ND[i]	No	ND	ND	ND
10	7.7	5	No	No changes	ND	46 ± 53
		4	No			7 ± 15
13	6.5	ND	No	ND	ND	ND
16	7.5	ND	No	ND	ND	ND
18	7.8	5	No	E, I484V; E, Q494G	NS1, E354E/K[k]; NS2A, V104M; NS4B, E7K	53 ± 17
		4	No			2 ± 16

[a] ChimeriVax-D2 virus was passaged in VeroPM cells (passages 141 to 147) at MOI of 0.1 to 0.5 and harvested 2 to 3 days postinfection.
[b] Titers ($\log_{10}$ PFU/ml) were measured in a plaque assay using VeroPM cells.
[c] $\log_{10}$ PFU/mouse.
[d] Groups of 4-week-old ICR females ($n = 5$) were inoculated by i.c. or s.c. routes.
[e] Amino acids of ChimeriVax-D2 (D2-PrME) envelope protein are compared to the published sequence for PUO-218 virus (7).
[f] Amino acids of ChimeriVax-D2 NS proteins are compared to the published sequence for YF 17D virus (22).
[g] Nucleotide sequences leading to these substitutions are already present in the plasmids and are not the result of posttransfectional mutations (8, 9).
[h] GMT were measured from the last dilution of sera (obtained after i.c. inoculation), which resulted in a 50% reduction in number of virus plaques.
[i] ND, not determined.
[j] Changes are given in the following format: protein, position, with the ChimeriVax-D2 amino acid to the right and the reference strain (PUO-218 or YF 17D) amino acid to the left.
[k] E/K, mixture of E and K.

PFU/ml) (18, 19). It is possible that anti-NS1 antibodies and/or memory cytotoxic T-lymphocyte responses to NS1 protein in the YF-immune group eliminated virus-infected cells and reduced viremia. Nevertheless, all animals seroconverted to the 2nd vaccine (ChimeriVax-D2 or wild-type dengue-2 virus), as determined by the presence of neutralizing antibodies 28 and 118 days after immunization (Table 8). Neutralizing antibody titers in dengue-2 virus-immunized animals were higher than those in monkeys immunized with ChimeriVax-D2 virus. This might have been due to a higher replication rate of the wild-type dengue-2 virus as was shown by the high magnitude of viremia in these animals. Viremia and neutralizing antibody titers comparable to those for ChimeriVax-D2 virus were obtained when the prME genes of dengue-1 (western Pacific) or dengue-2 (NGC) viruses were inserted into a dengue-4 virus backbone (14). Monkeys immunized with 5 $\log_{10}$ PFU of an intertypic D4-D1 or D4-D2 chimera showed little or no signs of viremia and were protected against homologous challenge. The levels of neutralizing antibodies in these monkeys were comparable to those produced by ChimeriVax-D2 and were generally lower than those in monkeys inoculated with wild-type dengue-1 or dengue-2 viruses (14).

Upon challenge, no viremia was detected in groups that received ChimeriVax-D2 or wild-type dengue-2 virus, whereas all animals that received either YF 17D or PBS became viremic, with mean peak titer of 3.5 to 4.7 $\log_{10}$ FFU and mean duration of 4 to 5 days (Tables 8 and 9). These data suggested that YF 17D preimmunity did not interfere with protection induced by ChimeriVax-D2 vaccine. However, further experiments employing a vaccine dose lower than 4.0 $\log_{10}$ PFU, larger numbers of animals, and different intervals between first and second immunizations are required to extend these observations. We are currently addressing this antivector immunity in rhesus monkeys using a tetravalent YF–dengue-1 to -4 vaccine and are planning to test a YF-JE$_{GMP}$ vaccine in human volunteers with or without preimmunity to YF 17D vaccine.

The genetic stability of any live viral vaccines intended for human use is especially important for RNA viruses due to their "quasispecies" nature, which is associated with a high mutation rate of viral RNA polymerase enzyme. ChimeriVax-D2 virus was passaged in VeroPM cells 18 times, and its full genome sequence was determined and compared to that of the passage 3 virus (ChimeriVax-derived vaccines will be produced at passage levels of 5 to 8). The envelope protein of passage 3 virus differed from that of its parent, PUO-218, at amino acids 484 (I versus V) and 494 (Q versus G). The mutation leading to the I484V substitution was most likely present in the plasmids, since MON310 virus constructed from these plasmids revealed the same substitution (8). The Q494G substitution was the result of an intentionally introduced *Nar*I restriction site at the coding sequence for the E-NS1 junction. No additional mutations were found in the inserted dengue prME genes after 18 passages. However, within the YF virus genes, both parent (G) and mutant (A) nucleotides were present at position 3524 in the passage 18-virus. These nucleotides would produce viruses with either E (parent) or K (mutant) amino acids at position 354 of the NS1 protein (Table 10). We did not attempt to determine at what specific passage these mutations had occurred. Nonetheless, we previously reported that a single mutation in the E gene of ChimeriVax-JE had occurred at a passage ≥10 (9). The fact that we did not find any mutation in the prME genes of ChimeriVax-D2 upon 18 passages in cell culture indicated that these genes might be more stable in chimeric dengue virus than in chimeric JE virus. Similarly, the E genes of dengue viruses passaged in different cell lines were shown to be more stable (accumulated fewer mutations) than those of JE viruses (A. Barrett [University of Texas, Medical Branch] personal communication).

To determine if heterozygosity in amino acid 354 of the NS1 protein would affect virus replication in VeroPM cells, the neurovirulence phenotype, or virus immunogenicity in mice, viruses at various passages (3, 5, 8, 10, 13, 16, and 18) were titrated and those from selected passages (3, 5, 10, and 18) were inoculated into mice by the s.c. or i.c. route. The titer of virus and its large plaque morphology were stable across 18 passages, indicating that there was no selective pressure to fix mutations, which would decrease or increase virus replication. None of the viruses from the passages tested were neuroviru-

lent for 4-week-old mice, and the titer of neutralizing antibodies did not differ significantly across various passages (Table 10).

In sum, the high replication rate in VeroPM cells, attenuation profile for monkeys, immunogenicity, and protective efficacy, as well as in vitro genetic stability, of ChimeriVax-D2 make it an appropriate vaccine candidate to be evaluated in humans. Today it is generally accepted that a dengue virus vaccine should contain all four serotypes, which should be administered simultaneously to avoid possible sensitization of vaccinees to severe forms (DHF and dengue shock syndrome) of dengue virus infections. We have constructed additional chimeric viruses containing prME genes of the other three dengue serotypes and are currently evaluating them in nonhuman primates.

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REFERENCES

1. **Brandt, W. E., R. D. Cardiff, and P. K. Russell.** 1970. Dengue virions and antigens in brain and serum of infected mice. J. Virol. **6:**500–506.
2. **Bray, M., R. Men, I. Tokimatso, and C. J. Lai.** 1998. Genetic determinant responsible for acquisition of dengue type 2 virus mouse neurovirulence. J. Virol. **72:**1647–1651.
3. **Calisher, C., N. Karabatsos, J. M. Dalrymple, R. E. Shope, J. S. Porterfield, E. G. Westaway, and W. E. Brandt.** 1989. Antigenic relationships among flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J. Gen. Virol. **70:**37–43.
4. **Chambers, T. J., A. Nestorowicz, P. W. Mason, K. H. Eckels, and C. M. Rice.** 1999. Yellow fever/Japanese encephalitis chimeric viruses: construction and biological properties. J. Virol. **73:**3095–3101.
5. **Deubel, V., R. M. Kinney, and D. W. Trent.** 1988. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue-2 virus, Jamaica: comparative analysis of the full-length genome. Virology **165:**234–244.
6. **Eckels, K. H., W. E. Brandt, V. R. Harrison, J. M. McCoun, and P. K. Russell.** 1976. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. Infect. Immun. **14:**1221–1227.
7. **Gruenberg, A., W. S. Woo, A. Biedrzycka, and P. J. Wright.** 1988. Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue virus type 2, New Guinea C and PUO-218 strains. J. Gen. Virol. **69:**1391–1398.
8. **Gualano, R. C., M. J. Pryor, M. R. Cauchi, P. J. Wright, and A. D. Davidson.** 1998. Identification of a major determinant of mouse neurovirulence of dengue virus type 2 using stably cloned genomic-length cDNA. J. Gen. Virol. **79:**437–446.
9. **Guirakhoo, F., Z. X. Zhang, T. J. Chambers, S. Delagrave, J. Arroyo, A. D. T. Barrett, and T. P. Monath.** 1999. Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax™-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. Virology **257:**363–372.
10. **Hahn, Y. S., R. Galler, T. Huhnkapiller, J. M. Dalrymple, J. H. Strauss, and E. G. Strauss.** 1988. Nucleotide sequence of dengue-2 RNA and comparison of the encoded proteins with those of other flaviviruses. Virology **162:**167–180.
11. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenge to molecular biology. Science **239:**476–481.
12. **Halstead, S. B.** 1989. Antibody, macrophage, dengue virus infection, shock and hemorrhage: a pathogenetic cascade. Rev. Infect. Dis. **11:**S830–S839.
13. **Harrison, V. R., K. H. Eckels, J. W. Sagartz, and P. K. Russell.** 1977. Virulence and immunogenicity of a temperature-sensitive dengue-2 virus in lower primates. Infect. Immun. **18:**151–156.
14. **Lai, C. J., M. Bray, R. Men, A. Cahour, W. Chen, H. Kawano, M. Tadano, K. Hiramatsu, I. Tokimatsu, A. Pletnev, S. Arakai, G. Shameem, M. Rinaudo.** 1998. Evaluation of molecular strategies to develop a live dengue vaccine. Clin. Diagn. Virol. **10:**173–179.
15. **Mason, P. W., P. C. McAda, T. L. Mason, and M. J. Fournier.** 1987. Sequence of the dengue-1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. Virology **161:** 262–267.
16. **Monath, T. P.** 1994. Dengue–the risk to developed and developing countries. Proc. Natl. Acad. Sci. USA **91:**2395–2400.
17. **Monath, T. P.** 1999. Yellow fever, p. 815–879. *In* S. Plotkin and W. Orenstein (ed.), Vaccines. W. B. Saunders and Company, Philadelphia, Pa.
18. **Monath, T. P., I. Levenbook, K. Soike, Z. X. Zhang, M. Ratterree, K. Draper, A. D. T. Barrett, R. Nichols, R. Weltzin, J. Arroyo, and F. Guirakhoo.** 2000. Chimeric yellow fever 17D-Japanese encephalitis virus vaccine: dose-response effectiveness and extended safety testing in rhesus monkeys. J. Virol. **74:**1742–1751.
19. **Monath, T. P., K. Soike, I. Levenbook, Z. X. Zhang, J. Arroyo, S. Delagrave, G. Myers, A. D. T. Barrett, R. E. Shope, T. J. Chambers, and F. Guirakhoo.** 1999. Recombinant, chimeric live, attenuated vaccine (ChimeriVax™) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) and the capsid and nonstructural genes of yellow fever (17D) is safe, immunogenic and protective in non-human primates. Vaccine **17:**1869–1882.

19a. **National Institutes of Health.** 1998. Guide for the care and use of laboratory animals, rev. ed. Department of Health and Human Services publication no. (NIH) 85-23. National Institutes of Health, Bethesda, Md.

20. **Putnak, R., D. A. Barvir, J. M. Burrous, D. R. Dubois, V. M. D'Andrea, C. H. Hoke, J. C. Sadoff, and K. H. Eckels.** 1996. Development of a purified, inactivated, dengue-2 virus vaccine prototype in Vero cells: immunogenicity and protection in mice and rhesus monkeys. J. Infect. Dis. **174:**1176–1184.
21. **Rice, C. M., A. Grakoui, R. Galler, and T. J. Chambers.** 1989. Transcription of infectious yellow fever virus RNA from full-length cDNA templates produced by in vitro ligation. New Biol. **1:**285–296.
22. **Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss.** 1985. Nucleotide sequence of yellow fever virus: implication for flavivirus gene expression and evolution. Science **229:**726–733.
23. **Rosen, L.** 1958. Experimental studies of New World monkeys with dengue and yellow fever. Am. J. Trop. Med. Hyg. **7:**406–410.
24. **Russell, P. K., and A. Nisalak.** 1967. Dengue virus identification by the plaque reduction neutralization test. J. Immunol. **99:**291–296.
25. **Sabin, A. B.** 1952. Research on dengue during World War II. Am. J. Trop. Med. Hyg. **1:**30–50.
26. **Sabin, A. B., and R. W. Schlesinger.** 1945. Production of immunity to dengue with virus modified by propagation in mice. Science **101:**640–642.
27. **Scott, R. M., K. H. Eckels, W. H. Bancroft, P. L. Summers, J. M. McCown, J. H. Anderson, and P. K. Russell.** 1983. Dengue-2 vaccine: dose response in volunteers in relation to yellow fever immune status. J. Infect. Dis. **148:**1055–1060.
28. **Simmons, J. S., J. H. St. John, and F. H. Reynolds.** 1931. Experimental studies of dengue. Philipp. J. Sci. **44:**1–251.
29. **Sumiyoshi, H., G. H. Tignor, and R. E. Shope.** 1995. Characterization of a highly attenuated Japanese encephalitis virus generated from molecularly cloned cDNA. J. Infect. Dis. **171:**144–151.
30. **Whitehead, R. H., V. Chaicumpa, L. C. Olson, and P. K. Russell.** 1970. Sequential dengue virus infections in the white-handed gibbon (*Hylobates lar*). Am. J. Trop. Med. Hyg. **19:**94–102.
31. **Wisseman, C. L., Jr., B. H. Sweet, E. C. Rosenzweig, and O. R. Eylar.** 1963. Attenuated living type 1 dengue vaccines. Am. J. Trop. Med. Hyg. **12:**620–623.