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Chimeric Dengue Type 2 (Vaccine Strain PDK-53)/Dengue Type 1 Virus as a Potential Candidate Dengue Type 1 Virus Vaccine

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We constructed chimeric dengue type 2/type 1 (DEN-2/DEN-1) viruses containing the nonstructural genes of DEN-2 16681 virus or its vaccine derivative, strain PDK-53, and the structural genes (encoding capsid protein, premembrane protein, and envelope glycoprotein) of DEN-1 16007 virus or its vaccine derivative, strain PDK-13. We previously reported that attenuation markers of DEN-2 PDK-53 virus were encoded by genetic loci located outside the structural gene region of the PDK-53 virus genome. Chimeric viruses containing the nonstructural genes of DEN-2 PDK-53 virus and the structural genes of the parental DEN-1 16007 virus retained the attenuation markers of small plaque size and temperature sensitivity in LLC-MK_2 cells, less efficient replication in C6/36 cells, and attenuation for mice. These chimeric viruses elicited higher mouse neutralizing antibody titers against DEN-1 virus than did the candidate DEN-1 PDK-13 vaccine virus or chimeric DEN-2/DEN-1 viruses containing the structural genes of the PDK-13 virus. Mutations in the envelope protein of DEN-1 PDK-13 virus affected in vitro phenotype and immunogenicity in mice. The current PDK-13 vaccine is the least efficient of the four Mahidol candidate DEN virus vaccines in human trials. The chimeric DEN-2/DEN-1 virus might be a potential DEN-1 virus vaccine candidate. This study indicated that the infectious clones derived from the candidate DEN-2 PDK-53 vaccine are promising attenuated vectors for development of chimeric flavivirus vaccines.

Dengue (DEN) virus type 1 to 4 (DEN-1 to DEN-4) are mosquito-borne pathogens of the genus *Flavivirus* (family *Flaviviridae*). The flavivirus genome is a single-stranded, positive-sense RNA approximately 11 kb in length. The genome organization is 5′ noncoding region (NCR)-capsid (C)-premembrane/membrane (prM/M)-envelope (E)-nonstructural protein 1 (NS1)-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′NCR. The viral structural proteins, C, prM/M, and E, and the nonstructural proteins, NS1 to NS5, are translated as a single polyprotein and processed by cellular and viral proteases (12, 49).

Transmitted by *Aedes aegypti* mosquitoes to humans, DEN viruses cause tens of millions of cases, ranging from dengue fever to the sometimes fatal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), in tropical and subtropical regions of the world every year (42). Epidemiologic studies have shown that individuals who experience a secondary infection with a DEN virus serotype that differs from that of the previous infection are at higher risk of developing DHF/DSS (21). Therefore, an efficacious tetravalent vaccine is needed to provide solid and long-term immunity against all four DEN virus serotypes. Four parental DEN virus serotypes (DEN-1 16007, DEN-2 16681, DEN-3 16562, and DEN-4 1036) were passaged in cell cultures to obtain attenuated vaccine candidates at Mahidol University, Bangkok, Thailand (51). Human clinical trials have been conducted in Thailand and the United States (4–6, 17, 48). These attenuated viruses are currently the most promising DEN virus vaccine candidates in terms of immunogenicity and safety in humans. The Mahidol vaccine candidates DEN-1 PDK-13, DEN-2 PDK-53, DEN-3 PGMK-30/FRhL-3, and DEN-4 PDK-48 viruses have 50% minimum infectious dose values of 10^4, 5, 3,500, and 150 PFU, respectively, in humans (4). The candidate DEN-2 PDK-53 virus vaccine, which has the lowest infectious dose in humans, is strongly immunogenic and has produced no untoward clinical symptoms. The DEN-1 PDK-13 virus vaccine, on the other hand, has a high infectious dose and has resulted in minimal reactogenicity with lower seroconversion rate in human trials (4). While only one immunization with DEN-2 PDK-53 virus was required to achieve 100% seroconversion, a DEN-1 PDK-13 virus booster was needed to achieve the same seroconversion rate.

An understanding of the attenuation markers of the candidate DEN-2 PDK-53 virus vaccine should permit engineering of improved DEN virus vaccines. For this purpose, infectious cDNA clones of DEN-2 16681 and PDK-53 viruses (25), as well as recombinant DEN-2 16681/PDK-53 viruses (10), have been constructed. The uncloned PDK-53 virus vaccine contains a mixture of two genotypic variants (25), designated PDK53-E and PDK53-V in this report. The PDK53-V variant contains all nine PDK-53 virus vaccine-specific nucleotide mutations, including the Glu-to-Val mutation at amino acid position NS3-250. The PDK53-E variant contains eight of the nine mutations of the PDK-53 vaccine and the NS3-250-Glu of the parental 16681 virus. Infectious cDNA clones have been constructed for both variants, and viruses derived from both clones were attenuated in mice (10, 25). The phenotypic markers of attenuation of DEN-2 PDK-53 virus, including small plaque size and temperature sensitivity in LLC-MK_2 cells, limited replication in C6/36 cells, and attenuation for newborn mice, are determined by mutations in nonstructural regions of

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the genome, including 5′NCR-57 C-to-T (16681-to-PDK-53), NS1-53 Gly-to-Asp, and NS3-250 Glu-to-Val (10). Chimeric viruses containing the structural genes of other DEN serotypes within the DEN-2 PDK-53 genetic background would be expected to retain these phenotypic markers of attenuation. Chimeric viruses expressing DEN-1, DEN-3, or DEN-4 virus structural genes within the genetic background of PDK-53 virus might assume improved and equivalent replication efficiency in humans and permit optimization of a tetravalent DEN virus vaccine. In this study, we engineered chimeric viruses containing the C-prM-E structural gene region of DEN-1 16007 virus into the genetic backgrounds of both DEN-2 PDK-53-E and PDK-53-V variants to develop an alternative DEN-1 virus vaccine candidate. To better understand the low immunogenicity of the DEN-1 PDK-13 virus, we also determined the full genome sequences of DEN-1 16007 and PDK-13 viruses.

MATERIALS AND METHODS

Viruses and cell cultures. Wild-type DEN-1 16007 and DEN-2 16681 viruses were available in the virus collection at the Centers for Disease Control and Prevention. DEN-1 16007 virus was recovered from the serum of a patient with DHF/DSS in 1964 in Thailand. The virus was isolated following three passages in grivet monkey kidney BS-C-1 cells and one passage in LLC-MK_2 cells, passaged twice in *Toxorhynchites amboinenis* mosquitoes, and then passaged in primary dog kidney (PDK) cells at the Center for Vaccine Development, Mahidol University, to derive the candidate DEN-1 PDK-13 virus vaccine (22, 51). A single LLC-MK_2 passage of this candidate vaccine virus (lot March 10, 1989) was used in this study unless otherwise mentioned. Following the aforementioned mosquito passages, the 16007 virus was passaged once in LLC-MK_2 cells for use in this study.

Viruses were grown in LLC-MK_2 and C6/36 cells in Dulbecco's modified minimal essential medium (DMEM) containing penicillin-streptomycin and 5% fetal bovine serum (FBS). Virus plaque titrations were performed in six-well plates of confluent Vero or LLC-MK_2 cells as described previously (35). The first 4-ml overlay medium—containing 1% SeaKem LE agarose (FMC BioProducts, Rockland, Maine) in nutrient medium (0.165% lactalbumin hydrolysate [Difco Laboratories, Detroit, Mich.]), 0.033% yeast extract [Difco], Earle's balanced salt solution, 25 mg of gentamicin sulfate [BioWhittaker, Walkersville, Md.] and 1.0 mg of amphotericin B [Fungizone; E. R. Squibb & Sons, Princeton, N.J.], per liter and 2% FBS)—was added after adsorption of the 200-μl virus inoculum for 1.5 h at 37°C. Following incubation at 37°C for 7 days, a second 2-ml overlay containing additional 80 μg of neutral red vital stain (GIBCO-BRL, Gaithersburg, Md.) per ml was added. Plaques were counted 8 to 11 days after infection.

Construction of chimeric D2/1 infectious clones. (i) pD2-16681-P48, pD2-PDK53-E48, and pD2-PDK53-V48 vectors. The three DEN-2 virus backbone vectors used for construction of the chimeric D2/1 clones were modified from the previously reported DEN-2 virus infectious clones (25). Clone pD2-16681-P48 was modified from pD2/IC-30P-A to contain cloning sites *Mlu*I and *Ngo*MIV at nucleotide positions (nt) 450 and 2380, respectively. The same cloning sites were introduced into both DEN-2 PDK-53 virus-specific clones, pD2/IC-130Vx-4 and -130Vc-K, and the modified clones were designated pD2-PDK53-E48 and pD2-PDK53-V48, respectively. Two cloning errors were found in the original pD2/IC-130Vx-4 and -130Vc-K at nt 6665 and 8840 (10). These defects were corrected in pD2-PDK53-E48 and -V48. The introduced *Ngo*MIV cloning site resulted in two nucleotide mutations (nt 2381 and 2382; TG to CC), which encoded a Val-to-Ala change at E-482. The nucleotide changes introduced at the *Mlu*I site were silent (25). The *Mlu*I site introduced at the C/prM junction was used to clone the prM-E genes of heterologous viruses. The prM-E constructs are not reported in this study.

(ii) Chimeric pD2/1-PP, -EP, -VP, -PV, -EV, and -VV. Two intermediate DEN-2 virus clones, pD2I-P and pD2I-E, were constructed by deleting the *Hpa*I (nt 2676) to *Xba*I (3′ terminus of viral genomic cDNA) fragments of pD2-16681-P48 and pD2-PDK53-E48, respectively. These intermediate clones were used to subclone DEN-1 virus-specific cDNA fragments. The cDNA fragments containing the C-prM-E genes of DEN-1 16007 or PDK-13 virus were amplified by reverse transcriptase-mediated PCR (RT-PCR) from DEN-1 virus RNA with primers DEN-Bgl.5NC (5′-TAGAGAGCAGATCTCTG-3′; conserved sequence in the 5′NCR of DEN virus genomes, underlined sequence is a *Bgl*II site) and cD1-2394.Ngo (5′-TGTGACCATGCCGGCTGCGATGCACATCACCGA-3′; underlined *Ngo*MIV site followed by complementary sequence near the 3′ end of the E gene of DEN-1 virus). Amplified fragments were cloned into the *Bgl*II-*Ngo*MIV sites of the intermediate pD2I-P and pD2I-E clones. Intermediate, chimeric D2/1 clones were sequenced to verify the accuracy of the inserted DEN-1 virus-specific cDNA. Fragments excised from the intermediate D2/1 clones with *Ssr*I (preceding the T7 promoter) and *Ngo*MIV were cloned into the full-genome-length DEN-2 vectors, pD2-16681-P48, pD2-PDK53-E48, and pD2-PDK53-V48. Six full genome-length chimeric D2/1 plasmids were constructed by inserting the C-prM-E gene region of DEN-1 16007 or PDK-13 virus into these three vectors (Fig. 1). The plasmids and their virus derivatives were designated as described in the legend of Fig. 1.

Recovery of recombinant viruses. All recombinant plasmids were grown in *Escherichia coli* XL1-Blue cells. Recombinant viral RNA was transcribed and capped with the cap analog m^7GpppA from 200 to 400 ng of *Xba*I-linearized cDNA and then transfected into 3×10^6 to 4×10^6 LLC-MK_2 or BHK-21 cells by electroporation (25, 30). Transfected cells were transferred to 75-cm^2 flasks in DMEM containing 10% FBS. Viral proteins expressed in the transfected cells were analyzed by indirect immunofluorescence assay. Virus-infected cells were fixed in ice-cold acetone for 30 min. DEN-1 and DEN-2 virus-specific monoclonal antibodies 1F1 and 3H5, respectively, were used in the assay, and binding was detected with fluorescein-labeled goat anti-mouse antibody. Viruses were harvested after 8 to 10 days and were then passaged in LLC-MK_2 cells once (D2-16681-P48; D2-PDK53-E48 and -V48; D2/1-PP, -EP, and -VP) or twice (D2/1-PV, -EV, and -VV) to obtain working seeds. D2/1-EV and -VV viruses were passaged a third time in LLC-MK_2 cells to obtain higher viral titers required for challenge or immunization of mice.

Characterization of the replication phenotypes of chimeric viruses in cell cultures. Plaque sizes were measured 10 days after infection in LLC-MK_2 cells. Mean plaque diameters were calculated from 10 plaques for each virus.

Viral growth curves were performed in 75-cm^2 flasks of LLC-MK_2 or C6/36 cells at a multiplicity of infection (MOI) of approximately 0.001. After adsorption for 2 h, 30 ml of DMEM (for LLC-MK_2 cells) or overlay nutrient medium (for C6/36 cells) containing 5% FBS and penicillin-streptomycin was added, and the cultures were incubated in 5% CO_2 at 37 or 29°C, respectively. Aliquots of culture medium were harvested at 48-h intervals for 12 days, adjusted to 12.5% FBS, and stored at −80°C prior to titration.

Temperature sensitivity was tested in LLC-MK_2 cells. Cells grown in two sets of 75-cm^2 flasks were infected and incubated as described for the growth curve study. One set of cultures was incubated for 8 days at 37°C; the other was incubated at 38.7°C. The ratio of virus titer at 38.7°C versus the titer at 37°C was calculated. Virus was designated as temperature sensitive if the virus titer at 38.7°C was reduced 60% or more relative its titer at 37°C.

Sequencing of viral cDNA. Viral RNA was extracted from virus seed as described previously (29) or by using a QIAmp viral RNA kit (Qiagen, Valencia, Calif.). DEN-1 virus-specific primers were based on the published data of the Singapore strain S275/90 (18). Five to seven overlapping viral cDNA fragments were amplified by RT-PCR with the Titan One-Tube RT-PCR system (Roche Molecular Biochemicals, Indianapolis, Ind.). Both strands of the cDNA amplicons were sequenced directly. For sequencing of the DEN-1 PDK-13 virus genome, template genomic RNA was extracted directly from a vial of the candidate DEN-1 PDK-13 vaccine (lot March 10, 1989). The 5′- and 3′-terminal sequences of the DEN-1 16007 and PDK-13 virus genomes were determined with a 5′ RACE (rapid amplification of 5′ cDNA ends) kit (GIBCO BRL) and by tailing the genomic RNA with poly(A) as described previously (25). Automated sequencing was performed as recommended on a PRISM 377 sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.).

Mouse studies. Litters of newborn outbred white ICR mice (colony maintained at the Centers for Disease Control and Prevention) were inoculated intracranially with 5,000 PFU of virus in a volume of 30 μl. They were observed daily for paralysis and death, and surviving mice were individually weighed once each week for 5 weeks.

Neutralizing antibody responses were tested in 3-week-old ICR mice in two experiments. They were inoculated intraperitoneally with 10^4 PFU of virus and were boosted with the same amount of virus 3 weeks (experiment 1) or 6 weeks (experiment 2) later. Mice were bled 2 days prior to the boost and 3 weeks after boosting.

Neutralization assays. Mouse serum samples were tested for neutralizing antibodies by serum dilution-plaque reduction neutralization test (PRNT) without addition of complement. Sixty PFU of DEN-1 16007 virus was incubated with equal volumes of serial twofold dilutions of heat-inactivated (56°C for 30 min) mouse serum specimens overnight at 4°C. Six-well plates of Vero cells were inoculated with the serum-virus mixtures and incubated at 37°C in a 5% CO_2 incubator for 1.5 h. Plates were then treated as described for the plaque titration protocol. Back titrations of the input DEN-1 16007 virus were included in quadruplicate in each assay. The neutralizing antibody titer was identified as the highest serum dilution that reduced the number of virus plaques in the test by 50% or greater.

RESULTS

Construction of chimeric DEN-2/DEN-1 viruses. To assess the potential of infectious cDNA clones derived from the two variants of DEN-2 16681 PDK-53 virus (PDK-53-E and PDK-53-V) to serve as vectors for vaccine development, we engineered chimeric DEN-2/DEN-1 cDNA clones (D2/1-EP, D2/1-VP, D2/1-EV, and D2/1-VV) containing the structural genes of wild-type DEN-1 16007 virus or its vaccine derivative, strain PDK-13, within the backbone of these two vectors (Fig. 1).

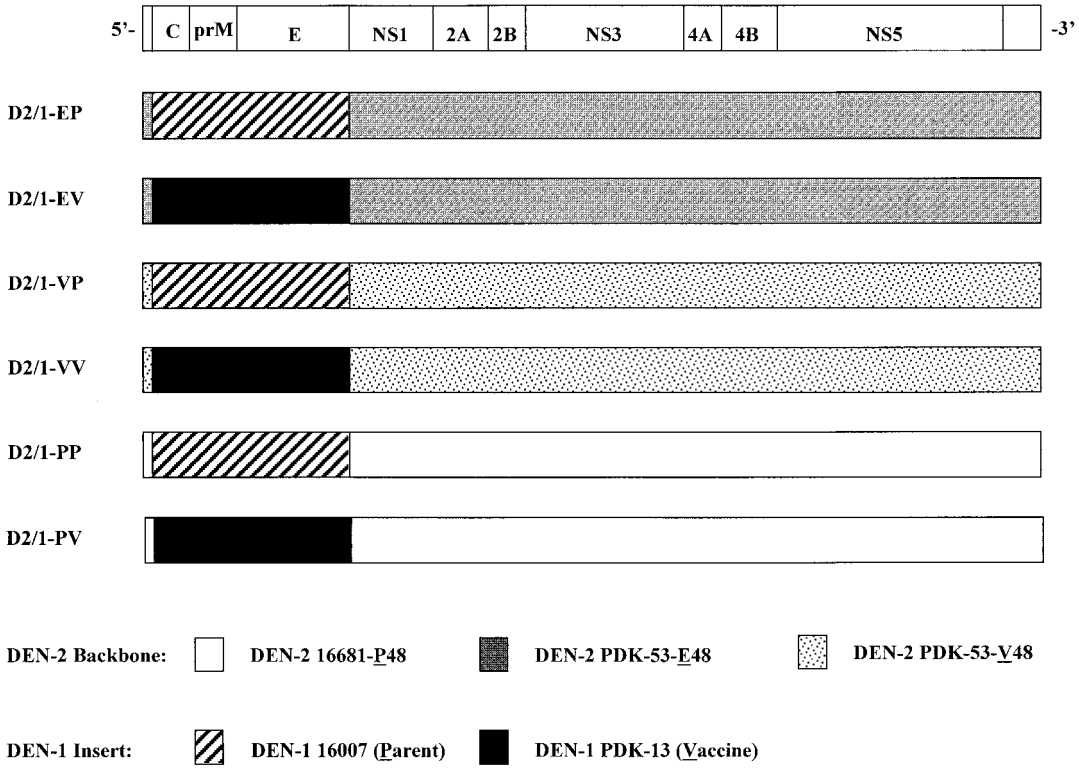

FIG. 1. Genomic organization of the chimeric DEN-2/DEN-1 viruses. Designations of the chimeras are based on the DEN-2 virus-specific infectious clone backbones and the structural genes (C-prM-E) insert of DEN-1 viruses. Underlined letters of the backbone and insert viruses are used in the designations. Designations: D2/1-*xy*, where *x* represents the infectious DEN-2 clone background (P, parental 16881 clone; E, PDK53-E variant; V, PDK53-V variant) and *y* denotes DEN-1 virus-specific C-prM-E insert (P, parental 16007 strain; V, PDK-13 vaccine candidate).

Two other chimeric clones, D2/1-PP and D2/1-PV, containing the structural genes of DEN-1 16007 or PDK-13 virus in the backbone of wild-type DEN-2 16681 virus, were also constructed for comparison (Fig. 1). We sequenced the entire full-length genomic cDNA in all of the infectious clones. A silent cDNA artifact was incorporated into the chimeric clones at nt 297 (T to C). A silent mutation at nt 1575 (T to C) was engineered into all of the chimeric clones to remove the natural *Xba*I site in the E gene of the DEN-1 virus.

Titers after transfection of LLC-MK_2 or BHK-21 cells were 10^4 to 10^6 PFU/ml for the chimeric viruses D2/1-PP, -EP, and -VP containing the C-prM-E of DEN-1 16007 virus. These titers increased to $10^{6.5}$ to $10^{7.5}$ PFU/ml after a single passage in LLC-MK_2 cells, comparable to the titers obtained for their parental viruses. Lower titers of 10^2 to 10^4 PFU/ml were obtained in transfected cells for the chimeric D2/1-PV, -EV, and -VV viruses containing the C-prM-E of DEN-1 PDK-13 virus. D2/1-PV virus reached 10^6 PFU/ml after two passages in LLC-MK_2 cells, whereas D2/1-EV and -VV viruses reached titers of $10^{3.3}$ to $10^{5.3}$ PFU/ml after two or three passages. Cells infected with any of the chimeric D2/1 viruses were positive by indirect immunofluorescence assay with monoclonal antibody 1F1 (specific for DEN-1 virus E protein) and negative with monoclonal antibody 3H5 (specific for DEN-2 virus E protein), indicating that appropriate DEN-1 virus E proteins were expressed by the chimeras (data not shown). The D2/1-PP, D2/1-EP, and D2/1-VP viral genomes were fully sequenced by directly analyzing overlapping RT-PCR fragments amplified from genomic viral RNA extracted from master seeds. All three genomes had the expected sequence.

Growth of the chimeric viruses in LLC-MK_2 and C6/36 cell cultures. All of the chimeric D2/1 viruses produced plaques smaller than the 6.8 ± 0.4-mm plaques of wild-type DEN-1 16007 virus in LLC-MK_2 cells (Fig. 2A). Both D2/1-EP (3.1 ± 0.3 mm) and D2/1-VP (2.8 ± 0.3 mm) virus plaques were similar in size to those of DEN-1 PDK-13 virus (2.9 ± 0.3 mm). The chimeric viruses D2/1-PV, D2/1-EV, and D2/1-VV containing the C-prM-E of DEN-1 PDK-13 virus formed tiny (1.3 ± 0.3 mm) or pinpoint (<1 mm) plaques. The D2-16681-P48 virus (Fig. 2A) produced 3.5 ± 0.3-mm plaques that were similar to plaques of wild-type DEN-2 16681 virus (data not shown). The D2-PDK53-V48 virus formed plaques that were smaller and fuzzier than those of the D2-PDK53-E48 virus. The 5.1 ± 0.3-mm plaques of D2/1-PP virus were larger than those of the other chimeric viruses but smaller than those of DEN-1 16007 virus.

Viruses were tested for temperature sensitivity in LLC-MK_2 cells and scored as indicated in Fig. 2B. As reported elsewhere (10), the DEN-2 PDK53-V variant (D2-PDK53-V48) was more temperature sensitive than DEN-2 PDK53-E virus (D2-PDK53-E48), and DEN-2 16681 virus (D2-16681-P48) was somewhat temperature sensitive (70 to 87% titer reduction at 38.7°C) (Fig. 2B). Multiple temperature sensitivity tests for D2-PDK53-E48 virus resulted in 83 to 97% growth reduction and an ambiguous score (+/2+ in Fig. 2B) because the cutting point between + and 2+ was set at 90% virus titer reduction at 38.7°C. The titer of DEN-1 16007 virus was reduced by 40% or less at 38.7°C, making it the least temperature sensitive virus in this study.

All of the DEN-1, DEN-2, and chimeric D2/1 viruses

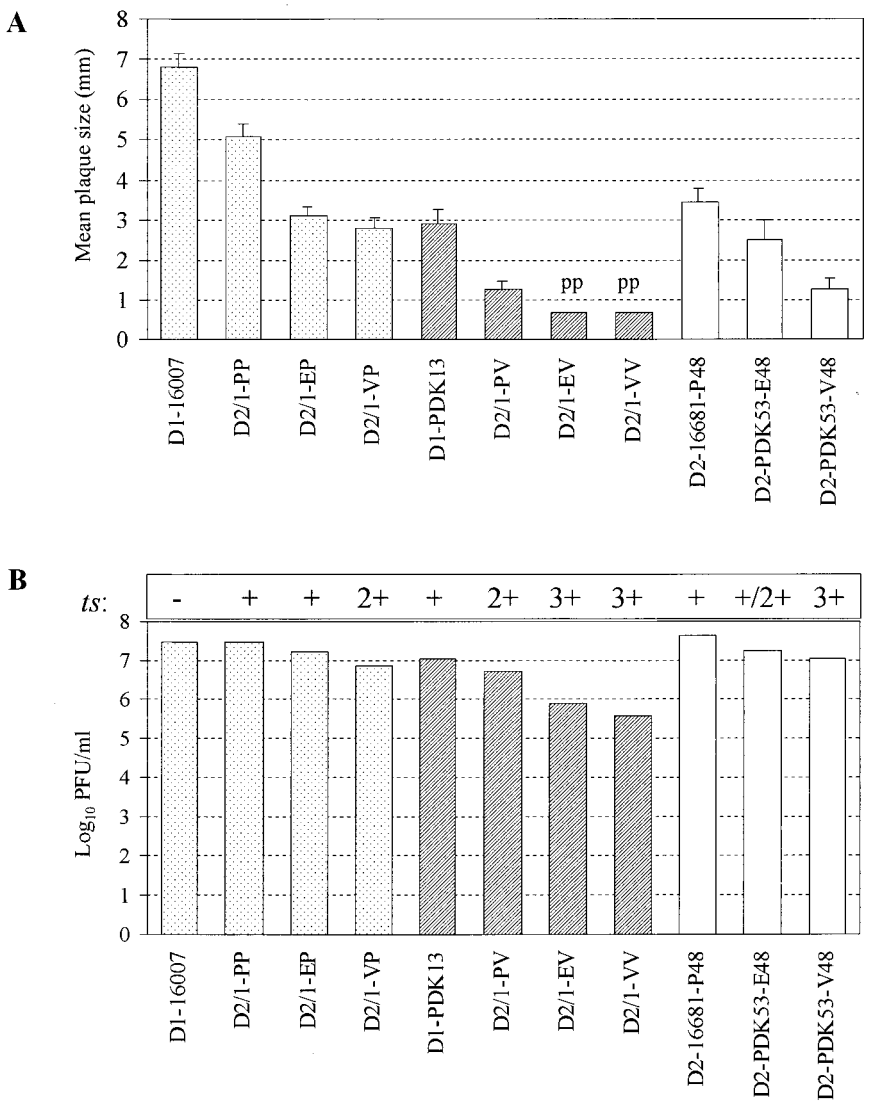

FIG. 2. Growth characteristics of the chimeric DEN-2/DEN-1 viruses in LLC-MK_2 cells. Stippled bars indicate DEN-1 16007 virus and the chimeric viruses expressing the structural proteins of DEN-1 16007 virus; gray bars indicate DEN-1 PDK-13 virus and the chimeric viruses expressing structural proteins of PDK-13 virus; blank bars indicate the three DEN-2 backbone viruses derived from infectious clones of DEN-2 16681 virus (P48) and the two variants (PDK53-E and PDK53-V; E48 and V48, respectively). (A) Mean (± standard deviation) plaque diameters. Values were calculated from 10 individual plaques of each virus on day 10 after infection. pp, pinpoint-size plaques less than 1 mm. (B) Temperature sensitivity (*ts*) and peak titers of chimeric viruses on day 8 or 10 after infection. The *ts* scores were based on the reduction of the virus titers at 38.7°C versus those at 37°C (−, +, 2+, and 3+ indicate titer reduction of ≤60, 61 to 90, 91 to 99, and >99%, respectively, calculated from at least three experiments). The graph bar heights represent the $\log_{10}$ titers of the viruses at 37°C. The MOI was approximately 0.001 PFU/cell.

reached peak titers between 8 and 10 days after infection in LLC-MK_2 cells (Fig. 2B). The clone-derived viruses D2-16681-P48, D2-PDK53-E48, and D2-PDK53-V48 (Fig. 2B) replicated to $10^{7.0}$ PFU/ml or greater, as did DEN-2 16681 and PDK-53 viruses (data not shown). Although reaching a similar peak titer, D2-PDK53-V48 virus replicated slower than the D2-PDK53-E48 virus during the first 4 days after infection (not shown). Chimeric D2/1-PP, D2/1-EP, D2/1-VP, and D2/1-PV viruses reached peak titers over $10^{6.7}$ PFU/ml, comparable to the peak titers of their parental DEN-1 and DEN-2 viruses. Chimeric D2/1-EV and -VV viruses, which had peak titers of $10^{5.6}$ to $10^{5.9}$ PFU/ml or lower in several separate experiments, replicated less efficiently than the other viruses.

Figure 3 shows viral growth curves in C6/36 cells. The three DEN-2 backbone viruses, D2-16681-P48, D2-PDK53-E48, and D2-PDK53-V48 (Fig. 3), replicated like the original DEN-2 16681 virus and the two PDK-53 variants (not shown), respectively. Both D2-PDK53-E48 and D2-PDK53-V48 viruses replicated about 4,000-fold less efficiently than the D2-16681-P48 virus in C6/36 cells (Fig. 3). Both DEN-1 16007 and PDK-13 viruses replicated to high titers of $10^{8.7}$ and $10^{8.4}$ PFU/ml, respectively. The chimeric D2/1-PP virus replicated to $10^{5.2}$ PFU/ml, which was equivalent to the peak titers of the two D2-PDK53 variants. Replication of chimeric D2/1-EP and D2/1-VP viruses was very inefficient in C6/36 cells. These viruses reached peak titers of lower than 10^2 PFU/ml. The replication of chimeric D2/1-PV, -EV, and -EV viruses was not tested in C6/36 cells.

Neurovirulence in suckling mice. Groups of newborn mice (n = 16) were inoculated intracranially with 5,000 PFU of virus. Wild-type DEN-2 16681 virus was 100% fatal, with an average survival time at 14.1 ± 1.6 days, while both clone-

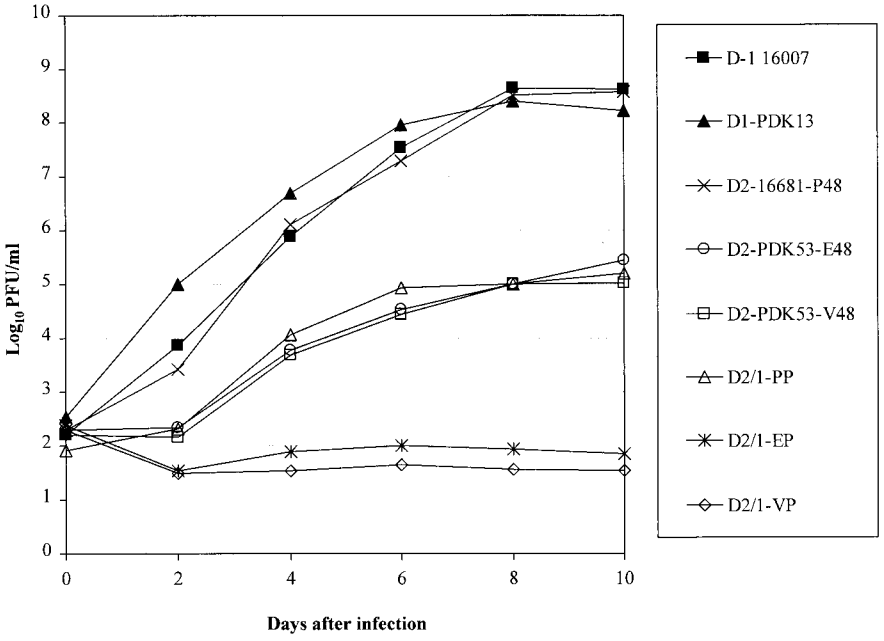

FIG. 3. Growth curves of DEN-1 16007, DEN-1 PDK-13, DEN-2 16681-P48, DEN-2 PDK53-E48, DEN-2 PDK53-V48, and chimeric DEN-2/DEN-1 viruses in C6/36 cells. Cells were infected at an approximate MOI of 0.001 PFU/ml.

derived D2-PDK53-E48 and D2-PDK53-V48 viruses failed to kill any mice (data not shown). Unlike DEN-2 16681 virus, which typically kills 50% or more of challenged mice (25), the wild-type DEN-1 16007 virus caused only a single fatality (21 days after challenge) in mice. The DEN-1 PDK-13 virus did not kill any mice (not shown). DEN-1 16007 virus-infected mice had significantly lower mean body weights ($P < 0.00003$, Student's t test), relative to the control group inoculated with diluent, between 21 and 35 days after challenge (Fig. 4). All of the mouse groups challenged with five chimeric D2/1 viruses (D2/1-VV virus was not tested) had mean weights lower ($P < 0.02$) than that of the control group but significantly greater ($P < 0.004$) than that of the DEN-1 16007 group 28 days after infection (Fig. 4). The mean body weights of mouse groups challenged with 10^4 PFU of DEN-1 16007 or PDK-13 virus (data not shown) were nearly identical to those of the mice challenged with 5,000 PFU of DEN-1 16007 virus (Fig. 4) between 7 and 35 days after challenge.

Immunogenicity of chimeric D2/1 viruses in mice. To test the immunogenicity of the chimeric viruses, groups of 3-week-old mice ($n = 8$) were immunized intraperitoneally with 10^4 PFU of virus in experiments 1 and 2 (Table 1). In experiment 1, mice were bled 20 days after primary immunization and then boosted 2 days later. In experiment 2, the mice were bled 41 days after primary immunization and boosted 2 days later. Table 1 shows the reciprocal, 50% plaque reduction endpoint PRNT titers of the pooled serum samples from each immunized group. The range of individual titers for the eight mice in many of the groups is also shown. In both experiments, the reciprocal titers of the pooled serum from 16007 virus-immunized mice were 80 before boost and 2,560 after boost. In both experiments, mice immunized with chimeric D2/1-PP, D2/1-EP, or D2/1-VP virus usually had a pooled serum titer that was at least as high as those of the 16007 virus-immunized groups before (reciprocal titers of 40 to 160) and after (reciprocal titers of 2,560 to 5,210) boost. The immune responses of the mice in these virus groups were nearly equivalent in experiments 1 and 2.

The PDK-13 virus and all three of the chimeras containing the PDK-13 virus structural genes induced minimal or low

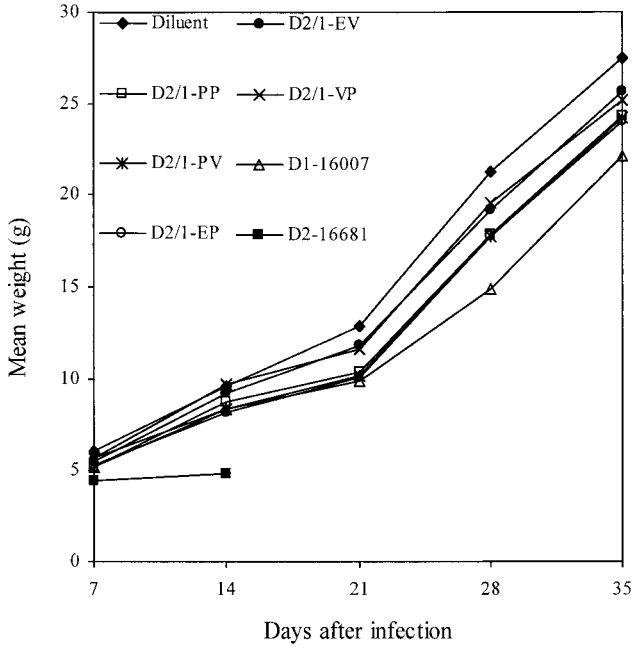

FIG. 4. Mean weights of mice inoculated intracranially with 5,000 PFU of DEN-1 16007, DEN-1 PDK-13, DEN-2 16681, or chimeric DEN-2/DEN-1 virus. No mice inoculated with DEN-2 16681 virus survived more than 18 days after challenge (average survival time of 14.1 ± 1.6 days), and one mouse died 21 days after inoculation with DEN-1 16007 virus.

TABLE 1. Immunogenicity of viruses in mice

Immunizing virus	PRNT titer[a] (range) against DEN-1 16007 virus in pooled sera[b]			
	Expt 1		Expt 2	
	Primary	Boost	Primary	Boost
DEN-1 16007	**80** (20–80)	2,560 (80–20,480)	80 (20–160)	2,560 (160–5120)
D2/1-PP	80	5,120	40 (10–160)	**5,120** (160–≥10,240)
D2/1-EP	80 (20–320)	10,240 (640–20,480)	160 (20–320)	5,120 (2,560–≥10,240)
D2/1-VP	40 (10–160)	2,560 (160–5,120)	**80** (10–320)	5,120 (40–≥10,240)
DEN-1 PDK-13	10 (10–40)	**80**	40 (20–80)	**320** (20–640)
D2/1-PV	10 (<10[c]–20)	80	40 (10–80)	2,560 (20–≥10,240)
D2/1-EV	20 (<10[c]–20)	80	40 (<10[c]–40)	**160** (10–320)
D2/1-VV	10 (10–40)	**80**	40 (10–160)	**160** (20–640)

[a] Reciprocal dilution yielding at least 50% plaque reduction. Where no range is given, individual titers were not determined. Boldface values indicate pooled sera titers fourfold higher than the titers calculated with 70% plaque reduction. All other pooled titers were either no different from or twofold higher than 70% plaque reduction titers.

[b] Three-week-old outbred ICR mice were immunized intraperitoneally with 10^4 PFU of virus and were boosted with the same virus dose 3 (experiment 1) or 6 (experiment 2) weeks later. Primary, serum taken 20 (experiment 1) or 41 (experiment 2) days after primary immunization; boost, serum taken 21 days after boost in both experiments.

[c] Only one mouse serum titer was less than 10 in this group.

reciprocal PRNT titers of 10 to 20 against DEN-1 16007 virus by 20 days after primary immunization in experiment 1. A somewhat higher reciprocal PRNT titer of 40 was elicited by each of these viruses by 41 days after immunization in experiment 2. The development of neutralizing antibodies was slower and of lower magnitude following immunization with PDK-13, D2/1-PV, D2/1-EV, or D2/1-VV virus than with 16007, D2/1-PP, D2/1-EP, or D2/1-VP virus in these mice. One mouse in the D2/1-PV group in experiment 1 and one mouse in each of the D2/1-EV groups in both experiments failed to produce a detectable PRNT titer before boost. Boosted titers were also higher for the PDK-13, D2/1-PV, D2/1-EV, and D2/1-VV-immunized groups in experiment 2 (pooled reciprocal titers of 160 to 2,560) than in experiment 1 (pooled reciprocal titers of 80). Except for the D2/1-PV group in experiment 2, these boosted titers were lower than the boosted PRNT titers induced by wild-type 16007 virus and chimeric D2/1-PP, -EP, and -VP viruses containing the structural genes of the wild-type DEN-1 16007 virus (Table 1). The high PRNT titer obtained for the pooled serum of the mice boosted with D2/1-PV virus resulted from two mouse sera which had reciprocal titers of 2,560 and ≥10,240. The remaining six mice in this group had reciprocal titers of 20 to 640, which were similar to the individual titers of mice in the PDK-13, D2/1-EV, and D2/1-VV groups. The PDK-13, D2/1-PV, D2/1-EV, and D2/1-VV viruses appeared to be less immunogenic than the 16007, D2/1-PP, D2/1-EP, and D2/1-VP viruses in these outbred mice. Pooled serum samples from mice immunized with D2-16681-P48, D2-PDK53-E48, or D2-PDK53-V48 virus did not contain detectable cross neutralizing antibody against DEN-1 16007 virus (not shown).

Nucleotide sequence analyses of DEN-1 16007 and PDK-13 virus genomes. We sequenced the genomes of wild-type DEN-1 16007 virus (GenBank accession no. AF180817) and its PDK-13 vaccine derivative (accession no. AF180818). There were 14 nucleotide and 8 encoded amino acid differences between 16007 and PDK-13 viruses (Table 2). Silent mutations occurred at nt 1567, 2695, 2782, 7330, and 9445 in the E, NS1, NS4B, and NS5 genes. Unlike the candidate DEN-2 PDK-53 vaccine virus, which has no amino acid mutations in the E protein (25), the DEN-1 PDK-13 virus had five amino acid mutations in E, including E-130 Val-to-Ala, E-203 Glu-to-Lys, E-204 Arg-to-Lys, E-225 Ser-to-Leu, and E-477 Met-to-Val. Amino acid mutations in the nonstructural genes included NS3-182 Glu-to-Lys, NS3-510 Tyr-to-Phe, and NS4A-144 Met-to-Val. The PDK-13 virus-specific E-477-Val was incorporated into all of the chimeric constructs.

DISCUSSION

In this report we compared chimeric viruses that expressed the structural genes of either wild-type DEN-1 16007 virus or its candidate PDK-13 vaccine derivative in the DEN-2 16681 and PDK-53 (both variants) genetic backgrounds. The D2/1-EV and -VV viruses, which contained the PDK-13 virus-specific C-prM-E, replicated to somewhat lower peak titers in LLC-MK_2 cells than did the other viruses in this study. These two viruses, as well as D2/1-PV virus, produced significantly smaller plaques and were more temperature sensitive in LLC-MK_2 cells than the chimeric viruses expressing the structural genes of DEN-1 16007 virus. The immunogenicity in mice of the three chimeric viruses expressing the C-prM-E of PDK-13

TABLE 2. Summary of nucleotide and amino acid differences between the genomes of DEN-1 16007 virus and its vaccine derivative, strain PDK-13

Position[b] (nt)	nt		Amino acid[a]		
	16007	PDK-13	16007	PDK-13	Position
1323	T	C	Val	**Ala**	E-130
1541	G	A	Glu	**Lys**	E-203
1543	A	G			
1545	G	A	Arg	**Lys**	E-204
1567	A	G	Gln	Gln	E-211
1608	C	T	Ser	**Leu**	E-225
2363	A	G	Met	**Val**	E-477
2695	T	C	Asp	Asp	NS1-92
2782	C	T	Ala	Ala	NS1-121
5063	G	A	Glu	**Lys**	NS3-182
6048	A	T	Tyr	**Phe**	NS3-510
6806	A	G	Met	**Val**	NS4A-144
7330	A	G	Gln	Gln	NS4B-168
9445	C	T	Ser	Ser	NS5-624

[a] Boldface indicates amino acid change.

[b] 1541 and 1543 were the first and third positions of a codon; mutations at both positions changed the codon for Glu to Lys at E-203.

virus, as well as the immunogenicity of PDK-13 virus itself, was somewhat reduced compared to the neutralizing antibody titers elicited by wild-type DEN-16007 virus and the three chimeras expressing the structural genes of 16007 virus. These data indicated that the mutations at E-130, E-203, E-204, and/or E-225, which constituted 50% of the amino acid substitutions in the translated polyprotein of PDK-13 virus, affected the plaque size, temperature sensitivity, and immunogenicity of the chimeric viruses, and probably contribute significantly to the phenotype of PDK-13 virus. These mutations, which resided in the dimerization domain II of the flavivirus E protein structure (40), may explain in part the high minimum infectious dose and low immunogenicity of PDK-13 virus in human vaccinees (4). The E-477-Val mutation, which occurred in the predicted TM2 transmembrane domain of the flavivirus E glycoprotein (1) and was incorporated into all of the chimeric viruses, did not adversely affect immunogenicity because the D2/1-PP, -EP, and -VP viruses were as highly immunogenic in mice as wild-type DEN-1 16007 virus. Other than serving as a membrane anchor and signal sequence for NS1, no other significant function has been ascribed to this TM2 domain (1).

Mutations in the nonstructural proteins of DEN-1 PDK-13 virus may also affect its phenotype. In particular, the NS3-182 Glu-to-Lys mutation of PDK-13 virus may be important because the NS3-182-Glu moiety of DEN-1 16007 virus is conserved as Glu or Asp in most mosquito-borne flaviviruses, although Japanese encephalitis (JE) and yellow fever (YF) viruses have other residues at this position (amino acid alignments not shown). The NS3 protein exhibits both protease and RNA helicase activities (14, 50), which may be affected by mutations in this protein, although the PDK-13 NS3-182 substitution was not located in the sequence motif for either of these two activities. The NS3-510 Tyr-to-Phe and NS4A-144 Met-to-Val mutations in PDK-13 virus might not be important attenuation markers. The NS3-510 locus is Phe in DEN-2, -3, and -4 viruses and two other strains of DEN-1 (Western Pacific 74 and Singapore S275/90) virus, while other flaviviruses contain a conservative Tyr at this position (not shown). The NS4A-144 mutation occurs at a locus that is Val, Leu, or Ile in other DEN virus serotypes (not shown). None of the mutations in the candidate DEN-1 16007/PDK-13 virus vaccine were shared with the candidate DEN-1 45AZ5/PDK-27 virus vaccine, which was derived from DEN-1 Western Pacific 74 virus by passage in diploid fetal rhesus lung cells, mutagenized with 5-azacytidine, and then serially passaged in PDK cells (39).

The PDK-13 virus-specific chimeras resulted in lower virus titers recovered from transfected cells relative to the 16007 virus-specific chimeras. Our experience with DEN-2/DEN-1 (this study) and DEN-2/DEN-4 (unpublished data) virus chimeras indicated that chimeric viruses which exhibited crippled replication during transfection and later developed high virus titers after passage in cell culture often accrued unexpected mutations. Viruses of increased replicative ability may arise through selection of subpopulations of virus variants, resulting from incorporation errors during in vitro transcription of cDNA (data not shown). Chimeric viruses that replicated well in transfected cells were more genetically stable after passage in LLC-MK_2 cells (not shown). Efficient replication with minimal passage in mammalian cell culture may be an important criterion of genetic stability and suitability for an infectious clone-derived vaccine virus.

Chimeric D2/1-PP virus, which contained the structural genes of DEN-1 16007 virus within the background of DEN-2 16681 virus, produced plaques that were smaller than those of DEN-1 16007 virus but larger than those of DEN-2 16681 virus. This indicated that the plaque phenotype of this chimeric virus was determined by both 16007 viral structural genes and the 16681 carrier background. The plaque sizes of chimeric D2/1-EP and D2/1-VP viruses in LLC-MK_2 cells were much smaller than those of DEN-1 16007 or D2/1-PP virus and about the same size as those of PDK-13 virus. All of the chimeric viruses were temperature sensitive, relative to DEN-1 16007 virus, and were at least as temperature sensitive as PDK-13 virus.

Reduced replication of DEN-2 PDK-53 virus in *A. aegypti*, relative to that of the parental DEN-2 16681 virus, may constitute a biological marker of attenuation of PDK-53 virus for humans (24). In this and previous studies (10, 25), both clone-derived DEN-2 PDK-53 variants replicated less efficiently than 16681 virus in *A. albopictus* C6/36 cells. Unlike the candidate DEN-2 PDK-53 vaccine virus, the candidate DEN-1 PDK-13 virus replicated with high efficiency that was nearly equivalent to that of its parental virus in C6/36 cells. The three chimeric D2/1-PP, -EP, and -VP viruses all replicated less efficiently than DEN-1 16007 virus in C6/36 cells. The approximately 4,000-fold reduction in replication of the D2/1-PP chimera, relative to wild-type DEN-1 16007 and DEN-2 16681 viruses, indicated a certain level of incompatibility between the replication machinery of DEN-2 virus and the structural genes of DEN-1 virus in C6/36 cells. It was not surprising that the replication of D2/1-EP and D2/1-VP viruses was reduced about 2,000-fold compared to D2/1-PP virus and 5 million-fold compared to the wild-type, parental DEN-2 16681, and DEN-1 16007 viruses. Their backbone D2-PDK53-E48 and D2-PDK53-V48 viruses replicated less efficiently than the wild-type DEN-1 and DEN-2 viruses in C6/36 cells. Other vaccine or candidate vaccine viruses—YF 17D, DEN-2 PR159/S-1, and JE 2-8—also replicate less efficiently and have lower oral infection and dissemination rates in *A. aegypti* or *Culex tritaeniorhynchus* than their parental viruses (2, 3, 15, 34, 45). A DEN-4 deletion mutant that failed to produce plaques in C6/36 cells was also replication defective in *A. albopictus* mosquitoes (11). DEN-1 PDK-13 virus has slightly lower infection (50% versus 60% for 16007 virus), dissemination (21% versus 43%), and in vitro transmission (13% versus 36%) rates in *A. aegypti* than the parental 16007 virus (23). Unlike the comparison between DEN-2 PDK-53 virus and 16681 parental virus (24), the different infection and transmission rates between DEN-1 PDK-13 and 16007 viruses were not statistically significant (23). Based on our results of viral replication in C6/36 cells, the D2/1-EP and D2/1-VP viruses would be expected to replicate less efficiently than DEN-1 PDK-13 virus in mosquitoes. The limited growth and dissemination of flaviviral vaccines in mosquitoes should limit the transmission of vaccine viruses from potentially viremic vaccinees (2, 15, 24, 34, 45). Attenuation in mosquitoes or mosquito cells not only may provide a biological marker for attenuated flavivirus vaccines but also is an important criterion for preventing natural secondary transmission of vaccine viruses.

The chimeric D2/1-EP and -VP viruses, which expressed the structural genes of wild-type DEN-1 16007 virus within the genetic backgrounds of the two DEN-2 PDK-53 variants, appeared to be potential DEN-1 vaccine candidates. These two chimeras replicated well in LLC-MK_2 cells and retained the attenuation markers associated with DEN-2 PDK-53 virus, including small plaque size, temperature sensitivity, restricted replication in mosquito cells, and attenuation for mice. They induced neutralizing antibody titers that were equivalent to titers elicited by wild-type DEN-1 16007 virus in mice. This suggests that the structural proteins of the 16007 virus, as expressed in the chimeric D2/1-EP, and -VP viruses, provided

optimal immunogenicity in these mice. On the other hand, DEN-1 PDK-13 virus replicated well in mosquito cells and was less immunogenic in outbred mice. Based on the degree of weight loss versus sham-inoculated control mice, the PDK-13 virus was similar to 16007 virus in the level of neurovirulence for newborn mice, while all of the tested chimeric DEN-2/DEN-1 viruses were somewhat less neurovirulent than 16007 virus in mice. The phenotypes of the chimeric D2/1-EP and D2/1-VP viruses, which differed at amino acid position NS3-250, were very similar in our study. This observation coincided with our previous demonstration that the 5′NCR-57 and NS1-53 loci were the dominant determinants of the attenuation markers of DEN-2 PDK-53 virus (10). However, we cannot rule out the possibility of differing infectivity and immunogenic efficacy of these vaccine candidates in humans. A monkey study is needed to determine which virus (D2/1-EP or -VP) might be the more effective vaccine candidate and if either one is equivalent to or more effective than the PDK-13 vaccine.

Chimeric flaviviruses have been investigated as potential vaccine candidates. Such viruses have been engineered to express the structural genes (C-prM-E or prM-E) of DEN-1, DEN-2, DEN-3, or tick-borne encephalitis virus within the genetic background of wild-type DEN-4 814669 virus (7, 8, 11, 16, 28, 33, 37, 38). Infectious clones of YF 17D (41) and DEN-2 PDK-53 (25) viruses were developed from vaccine or candidate vaccine strains that have been tested in humans. An attenuated, candidate chimeric vaccine virus containing the prM-E genes of the candidate JE SA14-14-2 vaccine virus within the genetic background of YF 17D virus has been constructed and shown to be safe and immunogenic in mice and rhesus monkeys (13, 19, 36). However, a YF 17D/JE chimera containing the prM-E of the virulent Nakayama strain of JE virus was neuroinvasive and neurovirulent in young adult mice (13, 19). The parental YF Asibi and 17D vaccine strains differ by 32 amino acids, including a substitution in prM and 12 substitutions in E (20). If some of the E mutations are involved in the attenuation of YF 17D virus, it is possible that 17D chimeras expressing structural genes of a virulent virus might not be attenuated.

Unlike the YF 17D vaccine, the DEN-2 PDK-53 virus has no amino acid substitutions in E, and the single prM-29 Val-to-Asp mutation has been shown to have minimal or no effect on the attenuation markers of PDK-53 virus (10). Yet this candidate vaccine virus is attenuated in mice and has been shown to be safe and immunogenic in human trials (4, 17, 48, 51). We have shown that both D2-PDK53-E and -V genetic backgrounds were sufficient to maintain markers of attenuation in chimeric D2/1-EP and -VP viruses that expressed the structural genes of wild-type DEN-1 virus. The strategy of using a genetic background that contains the determinants of attenuation in nonstructural regions of the genome to express the structural genes of heterologous viruses may enhance the development of live, attenuated flavivirus vaccine candidates that express wild-type structural genes of optimal immunogenicity. This strategy might be particularly useful in the design of vaccine candidates for immunogenic variants of a given flavivirus pathogen.

A live attenuated tetravalent DEN virus vaccine should provide life-long humoral and cellular immunity. Several DEN virus structural and nonstructural proteins are known to be targets for cytotoxic T-cell-mediated immune responses to DEN virus (9). The structural proteins appear to induce serotype-specific cytotoxic T lymphocytes (CTLs) (32, 43). Of the nonstructural proteins, NS3 appears to be a dominant source for both serotype-specific and serotype-cross-reactive CTL epitopes (27, 31, 43, 46). The serotype-cross-reactive DEN virus-specific CTL response induced in a primary infection is thought to play a role in the immunopathogenesis of DHF during a secondary DEN virus infection (43, 46). Activation of cross-reactive T cells may contribute to the pathogenesis of DHF via production of cytokine and cytolytic activities (26). The DEN-2 virus-specific nonstructural proteins in our chimeric DEN viruses present the possibility of inducing serotype-cross-reactive CTLs with potential risk of DHF for the vaccinee following a secondary infection. However, epidemiologic studies have suggested that the order of acquisition of DEN virus infections is important and that the risk of DHF/DSS is greatest if the agent of secondary infection is DEN-2 virus (44, 47). Studies of CTL in DEN virus-infected mice and humans suggest that complex patterns of CTL responses, including serotype-specific, subcomplex-specific, serotype-cross-reactive, and flavivirus-cross-reactive responses, are influenced by the viral serotype (43, 46). It has been suggested that CTLs induced by other DEN virus serotypes may recognize DEN-2 virus to a greater extent than the DEN-2-induced CTLs recognize DEN-1, -3, or -4 virus (46). The DEN virus-specific T-cell responses in DEN-2 PDK-53 virus-immunized vaccinees have been shown to be predominantly serotype specific (17). Therefore, the cross-reactive T-cell response induced by immunization with a DEN-2 PDK-53-based chimeric virus may not recognize other serotypes of DEN virus efficiently and therefore avoid DHF/DSS following infections with heterologous serotypes of DEN virus. In addition, a tetravalent vaccine formulated with the DEN-2 PDK-53 virus and PDK-53-based chimeric DEN-2/1, DEN-2/3, and DEN-2/4 viruses might reduce possible interference among the four vaccine viruses. Such interference might be more pronounced in a conventional tetravalent vaccine because of more extensive subcomplex-cross-reactive and serotype-cross-reactive CTL responses induced by the NS3 proteins of all four DEN virus serotypes. A PDK-53 virus-based tetravalent vaccine may help ensure that all four viruses replicate efficiently in the vaccinee to induce immunity against all four serotypes of DEN virus concurrently. We are now employing the strategy outlined in this study to develop chimeric DEN-2/3 and DEN-2/4 viruses.

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