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Yellow Fever Virus/Dengue-2 Virus and Yellow Fever Virus/Dengue-4 Virus Chimeras: Biological Characterization, Immunogenicity, and Protection against Dengue Encephalitis in the Mouse Model

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Two yellow fever virus (YFV)/dengue virus chimeras which encode the prM and E proteins of either dengue virus serotype 2 (dengue-2 virus) or dengue-4 virus within the genome of the YFV 17D strain (YF5.2iv infectious clone) were constructed and characterized for their properties in cell culture and as experimental vaccines in mice. The prM and E proteins appeared to be properly processed and glycosylated, and in plaque reduction neutralization tests and other assays of antigenic specificity, the E proteins exhibited profiles which resembled those of the homologous dengue virus serotypes. Both chimeric viruses replicated in cell lines of vertebrate and mosquito origin to levels comparable to those of homologous dengue viruses but less efficiently than the YF5.2iv parent. YFV/dengue-4 virus, but not YFV/dengue-2 virus, was neurovirulent for 3-week-old mice by intracerebral inoculation; however, both viruses were attenuated when administered by the intraperitoneal route in mice of that age. Single-dose inoculation of either chimeric virus at a dose of 10^5 PFU by the intraperitoneal route induced detectable levels of neutralizing antibodies against the homologous dengue virus strains. Mice which had been immunized in this manner were fully protected from challenge with homologous neurovirulent dengue viruses by intracerebral inoculation compared to unimmunized mice. Protection was associated with significant increases in geometric mean titers of neutralizing antibody compared to those for unimmunized mice. These data indicate that YFV/dengue virus chimeras elicit antibodies which represent protective memory responses in the mouse model of dengue encephalitis. The levels of neurovirulence and immunogenicity of the chimeric viruses in mice correlate with the degree of adaptation of the dengue virus strain to mice. This study supports ongoing investigations concerning the use of this technology for development of a live attenuated viral vaccine against dengue viruses.

The dengue group of viruses includes four serotypes (dengue-1 through dengue-4) that exhibit a high level of genome sequence homology and similar envelope protein antigenic properties (4, 8, 33, 58). Each virus can cause a primary infection in humans, classically known as dengue fever and characterized by a nonfatal febrile illness of variable severity, usually in older children or adults (15, 39). Infection with a single serotype confers protection against reinfection with homologous strains but does not provide long-lasting cross-protection against heterologous strains. Severe forms of dengue virus infection (dengue hemorrhagic fever and dengue shock syndrome) are believed to result in part from sequential infections with heterologous serotypes associated with antibody-dependent enhancement (ADE) of infection mediated by cross-reactive, nonneutralizing antibody (21, 22, 23, 30). ADE, in conjunction with activation of memory T-cell responses, is the factor which is believed to contribute to the immunopathogenic disease process (50).

There is a growing global burden of human disease due to the dengue viruses. It is estimated that there are as many as 50 to 100 million cases of dengue fever and several hundred thousand cases of dengue hemorrhagic fever worldwide annually, with an overall case fatality rate of ~5% (17, 40). The development of vaccine for dengue viruses is a major public health priority but remains problematic because of the need to elicit high levels of neutralizing antibodies against all four serotypes. A vaccine which achieves this is expected to provide uniform protection against all serotypes and a low risk of ADE. A variety of vaccine approaches have been undertaken, including empirically derived and cDNA-derived live attenuated viruses, recombinant subunit vaccines, inactivated virus, and DNA vaccines (2, 3, 5, 13, 19, 20, 24, 25, 28, 31, 32, 45, 47, 48, 52–54). Although some candidates have progressed to clinical trials, there have been problems with immunogenicity and reactogenicity of certain vaccines, and it is not yet known which modality will be most suitable for use in humans.

We have pursued the use of infectious-clone technology for producing live attenuated viruses composed of flavivirus structural antigens within the backbone of the yellow fever virus (YFV) 17D (YF17D) infectious clone. Investigation of this chimeric-virus approach for novel flavivirus vaccines has been promising, with the development of experimental vaccines for tick-borne encephalitis virus and dengue and Japanese enceph-

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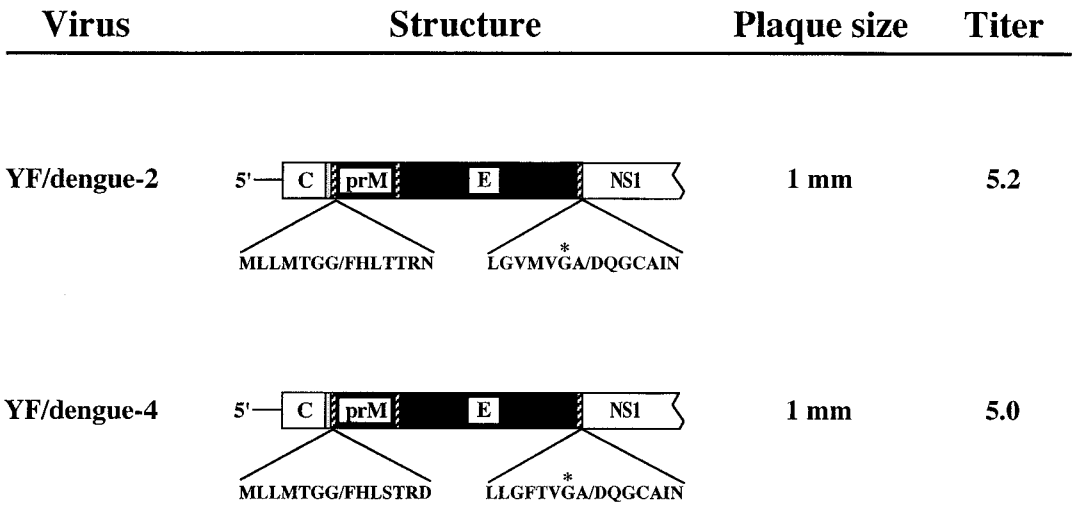

FIG. 1. Diagram of the template region encoding the chimeric structural proteins of YFV/dengue virus chimeras. The 5′ nontranslated region is derived from YF5.2iv (49). The open region represents the YFV capsid (C) protein. The solid regions represent dengue virus structural proteins (prM-E). The diagram is truncated within the YFV NS1 protein for clarity. The remainder of the genome is derived from YF5.2iv. The letters (single amino acid code) indicate the chimeric C/prM and E/NS1 junctions. The asterisks mark the residues changed from the native dengue-2 virus and dengue-4 virus sequences (glutamine to glycine at position P2 in both cases). The virus titers are given as $\log_{10}$ PFU per milliliter in the medium at time of harvest after RNA transfection of Vero cells, and the plaque sizes were determined by titration on Vero cells (see Results).

alitis viruses (5, 12, 18, 19, 42–44). With increasing knowledge of genetic determinants of virulence for dengue virus and other flaviviruses (6, 7, 29, 34, 36, 46), this approach in theory allows for modification of the vaccine to reduce or eliminate unacceptable neurovirulence or other undesirable tropisms. In the present study, we describe the properties of two YFV/dengue virus chimeras which we have characterized in cell culture and tested as experimental vaccines in laboratory mice. One of these viruses (YFV/dengue-2 virus), originally constructed in our laboratory, has been tested in detail in a rhesus monkey model for neurovirulence and protection against dengue virus challenge (19, 20). Here, we report additional studies of the structure, immunogenicity, and protective efficacy of this YFV/dengue-2 virus, as well as similar studies of a YFV/dengue-4 virus.

MATERIALS AND METHODS

Cells and viruses. BHK-21, Vero, and C6/36 cells were used as described previously (12). Dengue-2 virus (New Guinea C [NGC] strain, passage 2 in C6/36 cells, and neuroadapted suckling mouse brain-passaged dengue-2 virus NGC) was originally obtained from Walter Brandt. The NGC strain was used as a control dengue-2 virus in the various experiments in this study, and the neuroadapted virus was used as a challenge virus in mouse protection studies. Dengue-4 virus (VR-1257) was obtained from the American Type Culture Collection (ATCC) and passaged on Vero cells to produce a working stock of virus and RNA for cDNA cloning. Plaque assays were done by infection of Vero cell monolayers with dilutions of the various viruses at 37°C for 1 h, followed by removal of the inoculum and addition of 1% ME agarose (SeaKem) in alpha minimal essential media (MEM) plus 5% fetal bovine serum (FBS). Plaques were visualized by staining them with neutral red (0.05% in phosphate-buffered saline [PBS]), followed by fixation in 7% formalin, staining in crystal violet, and counting of plaques. Plaques were counted at 5 days for YFV, 6 days for dengue-2 virus, 7 days for YFV/dengue-2 virus, and 7 to 8 days for YFV/dengue-4 virus and dengue-4 virus. Titers of dengue-4 and YFV/dengue-4 viruses were also detected by fluorescence assay as described below. All PFU numbers in these experiments are based on Vero cell infectivity.

Plasmid constructions. A set of two-plasmid systems for generating full-length chimeric YFV/dengue-2 virus and YFV/dengue-4 virus templates for RNA transcription were constructed by engineering chimeric C/prM and E/NS1 junctions for both templates, as well as internal ligation sites in the dengue virus E protein coding regions. For the YFV/dengue-2 virus chimera, a plasmid used for construction of a dengue-2 virus infectious clone (pDVWS307 [16]) was used as a starting template for engineering the YFV/dengue-2 virus chimera. The virus encoded by pDVWS307 contains the prM-E region of the PUO-218 strain of dengue-2 virus, which was originally isolated from *Toxorhynchites splendens* mosquitoes and LLC-MK2 cells, and has had limited passages in C6/36 cells (16). The dengue-2 virus sequences in pDVWS307 were used to construct two YFV/dengue-2 virus plasmids containing the chimeric C/prM and E/NS1 junctions. The chimeric C/prM junction (Fig. 1) was engineered by PCR. Two primers (YF 78, corresponding to nucleotides [nt] 6625 to 6639 in pYF5′3′IV [49], and YF C-481, corresponding to YFV nt 463 to 481) were used with pYF5′3′IV to generate a 763-bp PCR product whose 3′ terminus ends at the C terminus of the YFV capsid protein. A second PCR product was generated from pDVWS307 using primer 465 (dengue-2 virus nt 465 to 485), whose 5′ terminus encodes the amino terminus of the dengue-2 virus prM protein, and primer 1501 (dengue-2 virus nt 1501 to 1522). The two PCR products were both digested with *Not*I and *Sph*I. They were then ligated in a three-fragment reaction with pYF5′3′IV, which had been digested with the same enzymes, to generate pYF5′3′IV/dengue-2(prM/E), in which ligation of the PCR fragments was intended to establish the chimeric C/prM junction. Analysis of the recombinant plasmids revealed that an authentic junction had not been created, so the plasmid was then repaired. A primer (5′-GTTACGTGTGGTTAGATGGAATCCACCCGTCATCAACAGC ATTCC-3′) (the underlining indicates a *Tfi*I site) containing the YFV sequence from nt 458 to 482 and the dengue-2 virus sequence from 465 to 485 was used as a 3′ primer, together with a 5′ primer (YF 78), to produce a 785-bp PCR product from pYF5′3′IV. A second PCR product of 1,073 bp was generated from pDVWS307 using a 5′ primer (5′-TTGTTGTCATTATTGATTCCATCTAAC CACACGTAACGG-3′) (the underlining indicates a *Tfi*I site) containing dengue-2 virus nt 465 to 487 in combination with a 3′ primer (dengue 1501). The PCR fragments were digested with *Not*I plus *Tfi*I and *Tfi*I plus *Bam*HI, respectively. They were then ligated together in a three-fragment reaction with pBS(KS+)II (Stratagene), which had been digested with *Not*I and *Bam*HI, to create pBS-YF/dengue-2(C/prM). The C/prM junction was formed by ligation at the *Tfi*I site. The chimeric YFV/dengue-2 virus region of this plasmid was placed back into the original pYF5′3′IV/dengue-2 in a three-piece ligation involving a PCR fragment (dengue-2 virus nt 465 to 1522) cut with *Sph*I and *Bst*EII, pBS-YF/dengue-2(C/prM) cut with *Not*I and *Bst*EII, and the pYF5′3′IV vector cut with *Not*I and *Sph*I. The integrity of the C/prM junction was verified by restriction enzyme digestion and nucleotide sequencing. The chimeric dengue-2 virus/YFV E/NS1 junction (Fig. 1) was engineered by PCR using a chimeric primer

(5′-GGAGTAATATGGCGCCCACCATAACTCCCAAAT-3′) containing dengue-2 virus nt 2425 to 2441 and a *Kas*I site (underlined) as a 3′ PCR primer, together with a 5′ primer corresponding to dengue virus nt 1289 to 1309, to produce a PCR product from pDVWS307. This was cloned into pYFM5.2[*Kas*I] (12), using *Kas*I and *Sph*I restriction sites, to yield pYFM5.2-YF/dengue-2. An extra *Sph*I site in pYFM5.2(*Kas*I) (YFV nt 6987) was eliminated by site-directed mutagenesis with incorporation of a silent nucleotide substitution (A for C at position 6898) to allow the use of a unique *Sph*I site in the dengue-2 virus envelope region for in vitro ligation of DNA templates.

Dengue-4 virus RNA was prepared from monolayers of virus-infected Vero cells by extraction with Trizol (Gibco/BRL). Oligonucleotide primers for reverse transcription and PCR amplification were designed on the basis of published dengue-4 virus nucleotide sequences (35, 59). cDNA synthesis was done using two minus-strand primers (5′-GATTGGATGTGTCATTTCC-3′ [dengue-4 virus nt 1392 to 1410] and 5′-GCTCGTTGGTTATTTGCTTCCACAT-3′ [dengue-4 virus nt 2619 to 2643]), together with Superscript II (Gibco/BRL), under conditions recommended by the manufacturer. The cDNA was amplified using the same reverse-transcription primers, together with the plus-strand primers 5′-G GGATTCTGAAGAGATGGGG-3′ and 5′-GGCAATGGCTGTGGCTTG-3′ (corresponding to dengue virus nt 288 to 307 and 1242 to 1259, respectively), in the presence of *Taq* DNA polymerase (Promega). The two PCR products were isolated by agarose gel electrophoresis, purified using Wizard PCR preps (Promega), and cloned into pCR-TOPO (Invitrogen) to produce pTOPO-DEN-4 (288 to 1410) and pTOPO-DEN-4 (1242-2643), respectively. To confirm that the cloned DNA encoded an intact open reading frame corresponding to the dengue-4 virus prM-E region, the nucleotide sequence was determined and compared to reported sequences for dengue-4 virus (27, 59, 60) (see Table 6). The pTOPO/DEN-4 (287-1392) clone was subsequently modified to extend the sequence to dengue virus nt 1932 by incorporation of an *Ava*I/*Sac*I fragment (nt 1301 to 1932) from pTOPO/DEN-4 (1242-2643). This was required to facilitate the construction of pYF5′3′IV/dengue-4 virus as described below. For dengue-4 virus, the C/prM junction (Fig. 1) was created by PCR using a chimeric primer (5′-ATCTCTTGTTGACAAGTGAAA**T**CCACCCGTCATCAACAGA-3′) (the boldface residues represent the chimeric junction, and the underlining indicates a *Hin*cII site), which was used as a 3′ primer, together with the 5′ primer YF 78, to amplify a 784-nt product from pYF5′3′IV. The fragment was cloned into pCR-Blunt-TOPO (Invitrogen), and subsequently, a *Not*I/*Hin*cII fragment from this plasmid was inserted into pBluescript-KSII(+) as a shuttle vector, using the same restriction sites. The *Not*I/*Hin*cII fragment was then cloned into pcrI-ITOPO/DEN-4 (288-1932) using the same restriction sites to produce pcrTOPO/DEN4(*Not*I-*Sac*I), with dengue-4 virus sequences from nt 441 to 1932. A 1,716-bp *Not*I/*Nsi*I fragment from this plasmid was then cloned into pYF5′3′IV, which had been digested with the same enzymes, to produce the final plasmid, pYF5′3′IV/DEN-4.

A fragment containing the chimeric dengue-4 virus/YFV E/NS1 junction (Fig. 1) was created by PCR using a primer encoding dengue-4 virus nt 2394 to 2414 and a *Kas*I site (underlined) (5′-ATGAATTAAAGGGCGCCAACTGTGAAA CCCAG-3′) as a 3′ PCR primer, together with a 5′ primer (5′-ACAATGGAG ACACGCATGCAGTAGGAAAT-3′ [dengue-4 virus nt 1369 to 1397]) to produce a 1,062-bp PCR product using pTOPO/DEN-4 (1241-2620) as a template in the presence of Vent DNA polymerase (New England Biolabs). The PCR product was cloned into pCR-Blunt-TOPO, and subsequently, an *Nsi*I/*Kas*I fragment was exchanged into pYFM5.2/JE-S (12) using the same restriction sites. Candidate clones of the final plasmids (pYFM5.2/DEN-4 and pYF5′3′IV/DEN-4) were screened by restriction enzyme digestions and nucleotide sequencing of the dengue-4 virus regions prior to use for regenerating virus.

RNA transcription and transfection. The sets of two-plasmid systems (YFM5.2/DEN-2 plus YF5′3′IV/dengue-2 and YFM5.2/DEN-4 plus YF5′3′IV/dengue-4) were used for construction of transcription templates by digestion with *Aat*II and *Sph*I (YFV/dengue-2 virus) or *Aat*II and *Nsi*I (YFV/dengue-4 virus), isolation of the appropriate restriction fragments, and ligation in vitro in the presence of T4 DNA ligase (New England Biolabs) essentially according to methods described previously (49). The ligated templates were linearized by digestion with *Xho*I, extracted with phenol-chloroform, and ethanol precipitated prior to being used for RNA transcription. RNA transcripts were synthesized using SP6 RNA polymerase (New England Biolabs) and transfected into Vero cells using Lipofectin (Gibco/BRL) as previously described (12). Typically, 100 ng of transcript was used for experiments to recover infectious virus. Transfected cell cultures were incubated until the onset of cytopathic effects (~4 to 6 days posttransfection), after which the media were harvested and yields of infectious virus were quantitated by plaque assay on Vero cells. Working virus stocks were prepared by amplification of the transfection harvest for one passage on Vero cells. The viruses were stored at −70°C prior to use in further experiments.

Nucleotide sequencing. Nucleotide sequencing was performed on the plasmids used for constructing the full-length cDNA templates (pYF5′3′IV/dengue-2, pYFM5.2/dengue-2, pYF5′3′IV/dengue-4, and pYFM5.2/dengue-4) using oligonucleotide primers designed based on the dengue virus sequences mentioned above. Reactions were run using Big Dye cycle-sequencing reaction kits (Perkin-Elmer/ABI, Inc.). Reaction products were analyzed using an Applied Biosystems DNA sequencer.

Growth and labeling of viruses. Growth curves were performed on confluent monolayers of C6/36, BHK, or Vero cells after infection at a multiplicity of 0.01 PFU/cell. The medium was harvested at 24-h intervals and replaced with fresh medium (alpha MEM plus 5% FBS). Virus yields were titrated by plaque assay on Vero cells at 37°C. For detection of labeled viral proteins, cells were either infected at the same multiplicity or mock infected and were labeled with [^{35}S]methionine (40 μCi/ml; Amersham) in methionine-free alpha MEM plus 5% FBS, beginning at approximately the peak of virus production based on the growth curve experiments. Tunicamycin (Sigma) was added at 10 μg/ml, or not added, to the labeling reaction. The cell monolayers were disrupted in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) in the presence of phenylmethylsulfonyl fluoride (20 μg/ml). The soluble proteins were then recovered after removal of the nuclei by centrifugation at 10,000 rpm for 10 min in an Eppendorf microcentrifuge. Viral proteins were immunoprecipitated using hyperimmune ascitic fluid against either dengue-2 or dengue-4 virus or YFV or nonimmune ascitic fluid. Immunoprecipitates were collected using Pansorbin (Calbiochem) and washed three times in lysis buffer plus 0.125% sodium dodecyl sulfate (SDS) and once in a solution of 150 mM NaCl–50 mM Tris-HCl, pH 7.5, followed by solubilization in SDS sample buffer, essentially as described previously (10). Proteins were analyzed on SDS polyacrylamide gels and visualized by fluorography.

Immunofluorescence. Detection of YFV/dengue-2 virus and YFV/dengue-4 virus chimeras and dengue-2 and dengue-4 viruses by immunofluorescence was done on Vero cell monolayers 48 h after low-multiplicity infection. The cells were fixed with 3% paraformaldehyde, permeabilized with 20% methanol, and treated with primary antibodies (mouse hyperimmune anti-YFV, anti-dengue-2 virus, and anti-dengue-4 virus [ATCC]), monoclonal antibodies specific for dengue-2 virus (anti-NGC envelope protein; clone 9.F.16) or dengue-4 virus (anti-H241 envelope protein; clone 9.F.22) (U.S. Biological), or rabbit polyclonal antisera to YFV E (10) or NS1 (51), followed by fluorescein isothiocyanate-labeled secondary antibodies (1:100 dilutions of either goat anti-mouse or goat anti-rabbit affinity-purified immunoglobulin G [ICN Pharmaceuticals]). Fluorescent cells were visualized using a Nikon TE-300 inverted microscope equipped with a fluorescein isothiocyanate fluorescence cube, and images were obtained using a SPOT camera (Diagnostic Instruments) and digital processing software supplied by the manufacturer.

Mouse experiments. Outbred (ICR) mice were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). The mice were handled as specified by institutional guidelines for care and use in accordance with Institutional Animal Care and Use Committee recommendations. For neurovirulence experiments, 4-week-old mice were inoculated by the intracerebral (i.c.) route with various doses of YFV (YF5.2iv infectious clone), dengue-4 virus, YFV/dengue-2 virus, or YFV/dengue-4 virus. The mice used for measurement of brain-associated YFV/dengue-2 virus were sacrificed between 3 and 13 days post-i.c. inoculation and perfused with PBS, and the brains were removed and homogenized as 20% (wt/vol) extracts in PBS plus 10% FBS. The virus content was measured by plaque assay on Vero cells.

For immunogenicity experiments, mice were inoculated with 10^5 PFU of either YFV/dengue-2 virus or YFV/dengue-4 virus diluted in PBS plus 10% FBS by the intraperitoneal route at 3 weeks of age. Groups of three or four mice were bled at weekly intervals from 4 to 8 weeks after immunization for measurement of neutralizing antibodies. Nonimmune sera were collected 6 to 8 weeks after mock immunization of a parallel group of mice which received an injection of PBS plus 10% FBS. Mock-immunized sera were pooled and used as a control in plaque reduction neutralization testing (PRNT). For protection experiments, mice were immunized as described above at 3 to 4 weeks of age and challenged between 5 and 6 weeks of age. Challenge was performed by i.c. inoculation of anesthetized mice with 10^4 PFU of parental dengue-4 virus (VR-1257) or 10^6 PFU of neuroadapted dengue-2 virus (suckling mouse brain preparation) diluted in PBS plus 10% FBS. The mice were observed until the onset of a moribund condition and then euthanized. Sera were collected from surviving and moribund mice for measurement of neutralizing-antibody titers.

Plaque neutralization assay. PRNT was done by incubation of serial twofold dilutions of test sera obtained from the immunized or mock-immunized mice with ~100 PFU of homologous dengue virus for 10 to 12 h at 4°C, followed by plaque assay on Vero cells. Positive control sera consisted of anti-dengue-2 virus

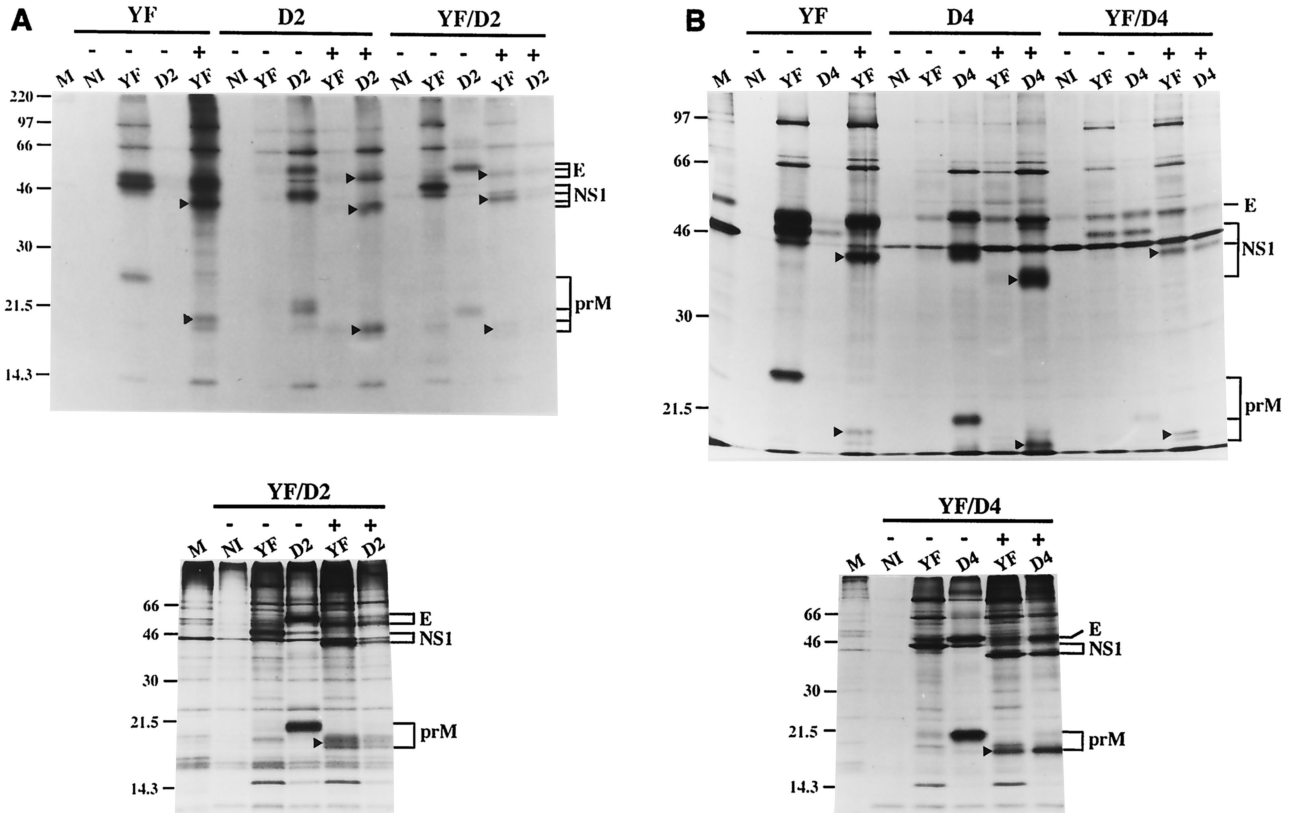

FIG. 2. Profiles of proteins of YFV/dengue virus chimeras. Parental or chimeric viruses were grown in BHK cells and labeled for 6 h with [^{35}S]methionine ~48 h postinfection, and viral proteins were immunoprecipitated from the cells as described in Materials and Methods. (A) Results for YFV, dengue-2 virus, and YFV/dengue-2 virus. Minus and plus indicate the absence or presence of tunicamycin in the labeling reaction. Lanes: M, mock-infected cells immunoprecipitated with a combination of YFV and dengue-2 virus or dengue-4 virus antisera; NI, nonimmune ascitic fluid; YF and D2, antisera to YFV and dengue-2 virus, respectively. (B) Results for YFV, dengue-4 virus, and YFV/dengue-4 virus. The lanes are as described for panel A, except that D4 indicates antiserum to dengue-4 virus. The proteins were analyzed on 11% (top of each panel) or 13% (bottom of each panel) SDS-polyacrylamide gels. The arrowheads indicate the positions of the unglycosylated E, NS1, and prM proteins. The multiple forms of the viral proteins E, NS1, and prM are indicated at the right. Molecular size markers are indicated at the left.

or anti-dengue-4 virus hyperimmune ascitic fluid. All sera were heated to 56°C for 30 min and then cooled to 4°C prior to addition of the virus. The plaque assay for dengue-4 virus was later replaced with a microfluorescence assay to improve reliability and provide more rapid results, since the standard plaque assay for this virus proved to be difficult to perform routinely and often required incubations for as long as 8 days. For fluorescence assays, Vero cell monolayers were infected with the serum-virus inoculum of dengue-4 virus and incubated for 72 h, followed by processing for fluorescence as described above and counting of fluorescent foci.

Neutralization assays to confirm the antigenic specificities of the chimeric viruses were done using similar methods. Approximately 100 PFU of YFV (YF5.2iv), YFV/dengue-2 virus, YFV/dengue-4 virus, dengue-2 virus, or dengue-4 virus was tested against serial twofold dilutions of hyperimmune ascitic fluid against YFV, dengue-2 virus, or dengue-4 virus, using nonimmune ascitic fluid as a negative control. Plaque counting for YF5.2iv, YFV/dengue-2 virus, and dengue-2 virus was done by plaque assay as described earlier. Plaque counting for YFV/dengue-4 virus and dengue-4 virus was done by fluorescence assay as described above.

RESULTS

Engineering and recovery of chimeric viruses. The general strategy for construction of the YFV/dengue virus chimeras followed that already successful for YFV/Japanese encephalitis virus chimeras (12). The prM-E region of either dengue-2 or dengue-4 virus was inserted in place of the corresponding region of the YF5.2iv virus, directly juxtaposing the authentic carboxy terminus of the YFV capsid protein with the amino terminus of the dengue virus prM proteins and creating an E/NS1 junction in which a signalase site is preserved and the carboxy termini of the dengue-2 and dengue-4 virus E proteins are modified by substitution of glycine for glutamine at the P2 position (Fig. 1). Transcription templates generated from the two plasmid systems for YFV/dengue-2 virus and YFV/dengue-4 virus produced infectious RNA transcripts that gave rise to virus within 5 days of transfection of Vero cells. Transfection of ~100 ng of RNA led to the recovery of ~5.0 to 6.0 log units (PFU/ml) of virus in typical experiments. The plaque sizes of the chimeric viruses on Vero cells were 1 mm for YFV/dengue-2 virus after 7 days of growth and 1 mm for YFV/dengue-4 virus after 8 days of growth.

Characterization of dengue virus proteins. To determine whether processing and glycosylation of the prM and E proteins of YFV/dengue virus appeared similar to those of homologous dengue viruses, lysates of virus-infected, radiolabeled cells were immunoprecipitated with antiserum to YFV or dengue-2 or dengue-4 virus. Figure 2 shows the results of these experiments. Antiserum to YFV immunoprecipitated the E

protein of YFV (50 kDa), the NS1 protein (48 kDa), and prM (24 kDa). Upon treatment with tunicamycin, the mass of the E protein was unchanged, whereas the NS1 and prM proteins migrated with apparent masses of 40 and 20 kDa, respectively. These results are consistent with previous studies showing that the E protein is nonglycosylated and that NS1 and prM each contain two N-linked oligosaccharides (10). Proteins consistent in mass with YFV NS5 (103 kDa), NS3 (70 kDa), and NS2B (14 kDa) were also immunoprecipitated. Antiserum to dengue-2 virus immunoprecipitated the E and NS3 proteins of YFV only very weakly. Antiserum to dengue-2 virus detected the E protein of dengue-2 virus (58 kDa) and proteins consistent in mass with NS1 (45 kDa) and prM (21 kDa). Upon treatment with tunicamycin, the mass of the E protein was 53 kDa, and the masses of the NS1 and prM proteins were 42 and 19 kDa, respectively. These masses are consistent with the presence of one or two N-linked oligosaccharides on the dengue-2 virus E protein and at least one each on the NS1 and prM proteins (11, 55). The dengue-2 virus antiserum also reacted with dengue virus NS5 (105 kDa), NS3 (69 kDa), and NS2B (14 kDa). Antiserum to YFV reacted only very weakly with the dengue-2 virus E, NS1, and prM proteins but was more reactive with the NS3 and NS5 proteins. Antiserum to dengue-2 virus immunoprecipitated the E protein of the YFV/dengue-2 virus, which migrated with the same apparent mass as the dengue-2 virus E protein in the presence and absence of tunicamycin (Fig. 2A, bottom). The antiserum also recognized a prM protein, which comigrated with the dengue-2 virus prM protein in the presence and absence of tunicamycin. This antiserum did not react with a protein consistent with NS1. In contrast, antiserum to YFV did react with a protein which migrated with the same apparent mass as the YFV NS1 protein in the presence and absence of tunicamycin. The prM protein was not recognized well by antiserum to YFV. The nonglycosylated dengue-2 virus E and prM proteins appeared to be recognized by the YFV antiserum better than the glycosylated form. YFV antiserum also recognized the NS3 and NS5 proteins of YFV/dengue-2 virus, whereas dengue 2 antiserum reacted well only with NS3.

For analysis of dengue-4 virus and YFV/dengue-4 virus, results with antiserum to YFV against YFV proteins were similar to those described above (Fig. 2B). Antiserum to dengue-4 virus reacted weakly with both the YFV E and NS1 proteins but not with the prM protein. Antiserum to dengue-4 virus reacted with a dengue-4 virus protein (52 kDa), which did not change in the presence of tunicamycin, suggesting a lack of N-linked glycosylation. The antiserum also reacted with the dengue-4 virus NS1 protein (42 kDa) and a 20-kDa protein presumed to be prM. The apparent masses of the NS1 and prM proteins in the presence of tunicamycin were 39 and 18 kDa, respectively, consistent with the presence of at least one N-linked oligosaccharide on each protein (37, 60). Dengue-4 virus proteins consistent in mass with NS5 (100 kDa), NS3 (69 kDa), and NS2B (14 kDa) were immunoprecipitated by the dengue virus antiserum. Antiserum to YFV reacted weakly with the dengue-4 virus E protein and the other dengue virus proteins. For the YFV/dengue-4 virus, antiserum to dengue-4 virus immunoprecipitated the E protein, which migrated with the same apparent mass as the dengue-4 virus E protein in the presence and absence of tunicamycin. This antiserum also reacted weakly with proteins consistent in mass with YFV NS1 and dengue-4 virus prM. These proteins underwent changes in apparent masses in the presence of tunicamycin that were similar to those of the YFV NS1 and dengue-4 virus prM proteins. Antiserum to YFV reacted with the E, prM, and NS1 proteins of YFV/dengue-4 virus. Reactivity with the unglycosylated form of prM appeared to be greater than that with the glycosylated forms. Taken together, these results indicate that the structural proteins prM and E of the chimeric viruses resemble those of the homologous dengue viruses from which they were derived during production in infected BHK cells. There was a small degree of cross-reactivity of the various antisera to the dengue virus and YFV E proteins that presumably reflects group-reactive epitopes. These were more apparent between YFV and dengue-4 virus than between YFV and dengue-2 virus. Cross-reactivity with the NS1 and prM proteins was less apparent than with E. Nonimmune antisera recognized only background cellular proteins in these experiments.

Immunofluorescence. Virus-infected Vero cells were used to characterize the reactivities of various antisera with proteins produced by the chimeric viruses and to establish the presence of serotype-specific epitopes on the E proteins of YFV/dengue-2 virus and YFV/dengue-4 virus. Figure 3 shows the results of these experiments. Antiserum to YFV or dengue-2 or dengue-4 virus reacted with all three viruses (data not shown). These results presumably reflect the presence of cross-reactive epitopes on E and possibly other proteins, which is consistent with the results of the immunoprecipitation experiments. Antiserum to YFV E protein at a dilution of 1:1,000 recognized YFV-infected cells but not YFV/dengue-2 virus- or YFV/dengue-4 virus-infected cells (Fig. 3, top row). Monoclonal antibody to dengue-2 virus recognized YFV/dengue-2 virus-infected cells but not cells infected with YFV or YFV/dengue-4 virus. In a similar manner, monoclonal antibody to dengue-4 virus recognized YFV/dengue-4 virus-infected cells but not cells infected with the other viruses. Polyclonal antiserum to YFV NS1 (1:1,000 dilution) was used to verify the presence of replicating virus in these experiments. This antiserum reacted with cells infected with YFV, as well as YFV/dengue-2 virus and YFV/dengue-4 virus. It did not react with dengue-2 or dengue-4 virus at the dilution used (data not shown).

Antigenic properties of chimeric-virus E proteins. PRNT was done to demonstrate the preservation of serotype-specific dengue virus epitopes on the E proteins of the chimeric viruses. The YF5.2iv, dengue-2, and dengue-4 viruses and the two chimeric viruses were each tested against mouse hyperimmune ascitic fluid to YF17D, dengue-2, or dengue-4 virus. Table 1 shows the results of these experiments, in which 90% plaque reduction titers were used to define the specificity of neutralization. Background neutralization from nonimmune ascitic fluid was not detectable at dilutions as low as 1:10 to 1:20 for any of the viruses. YFV ascitic fluid neutralized YF5.2iv virus to endpoint dilutions of 1:8,000 but did not neutralize either dengue virus or the chimeric viruses above the background level (1:10 or 1:20 dilutions). Antibody to either dengue-2 or dengue-4 virus did not show any significant neutralizing activity toward YF5.2iv. Dengue-2 virus ascitic fluid neutralized dengue-2 virus with an endpoint dilution of 1:1,000 and had activity against YFV/dengue-2 virus to a similar extent (1:1,000 dilution). This antibody also partially neu-

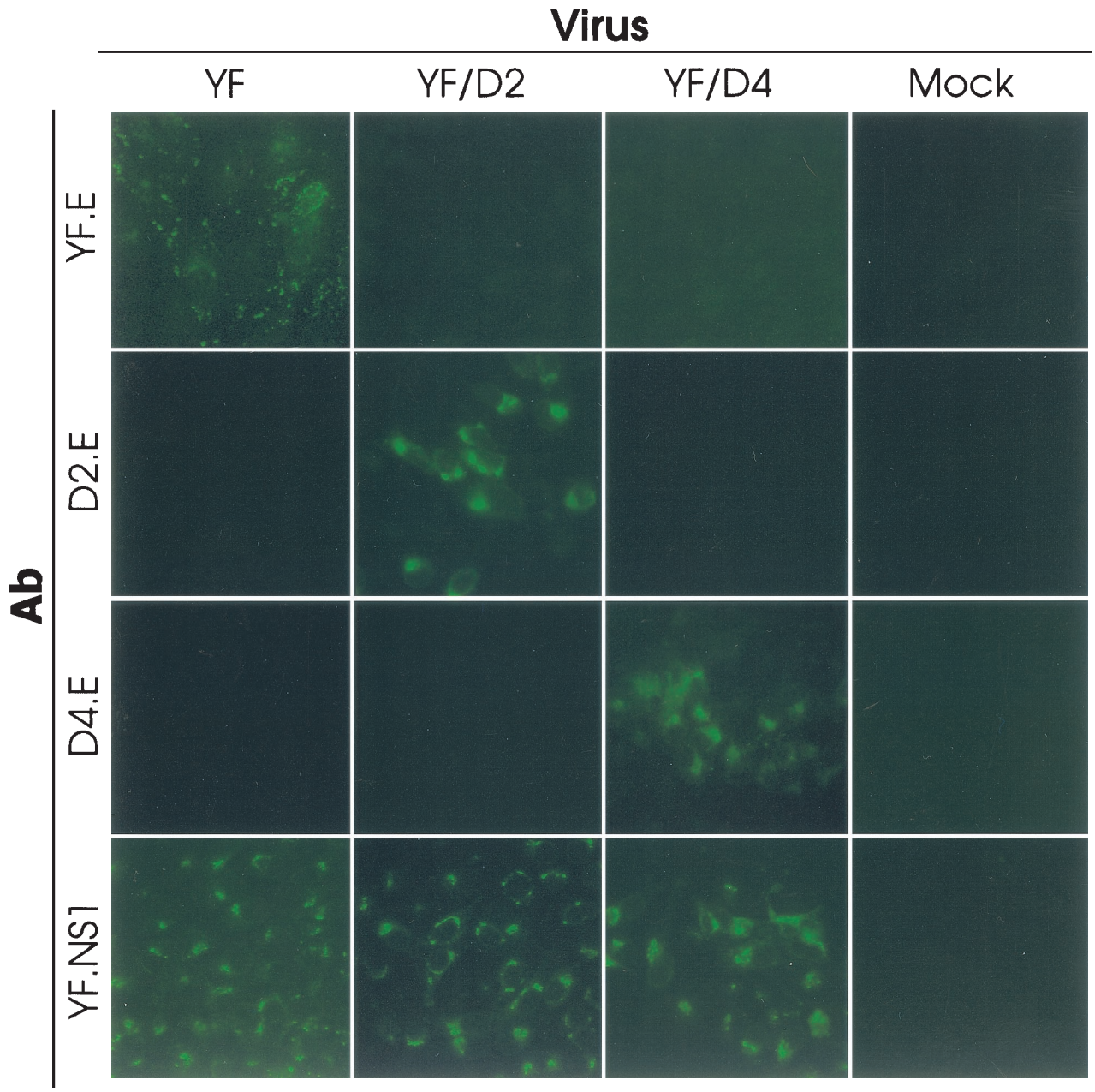

FIG. 3. Immunofluorescence of YFV (YF) and YFV/dengue (YF/D2 and YF/D4) viruses in Vero cells. Experiments were done as described in Materials and Methods. Top row, results with polyclonal antisera to YFV E protein (1:1,000 dilution); second and third rows, results with monoclonal antibodies to dengue-2 or dengue-4 virus (1:200 dilution); bottom row, results with polyclonal antisera to YFV NS1 protein (1:1,000 dilution).

tralized dengue-4 virus (endpoint dilution, 1:200) and YFV/dengue-4 virus (1:400 dilution). Antibody to dengue-4 virus neutralized dengue-4 virus and YFV/dengue-4 virus with endpoint dilutions of 1:2,000 and 1:4,000, respectively. However, it did not neutralize dengue-2 virus or YFV/dengue-2 virus (endpoint dilutions, <1:10 and <1:10, respectively). These experiments indicated that there was some cross-neutralization of dengue-2 virus by dengue-4 virus antibody, although the specificity of the latter antibody for its homologous virus was evident in a fivefold-higher neutralization titer. The neutraliza-

TABLE 1. Antigenic specificities of chimeric viruses

Virus	Antibody specificity[a]			
	NI	YF17D	Dengue-2	Dengue-4
YF5.2iv	<1.0	3.9	<1.0	<1.0
Dengue-2	<1.0	<1.0	3.0	<1.0
YFV/dengue-2	<1.0	<1.0	3.0	<1.0
Dengue-4	<1.3	<1.3	2.3	3.3
YFV/dengue-4	<1.0	<1.0	2.6	3.6

[a] Antibody refers to hyperimmune ascitic fluid to respective viruses. NI indicates nonimmune ascitic fluid. All values are log reciprocals of the 90% plaque reduction neutralization titer.

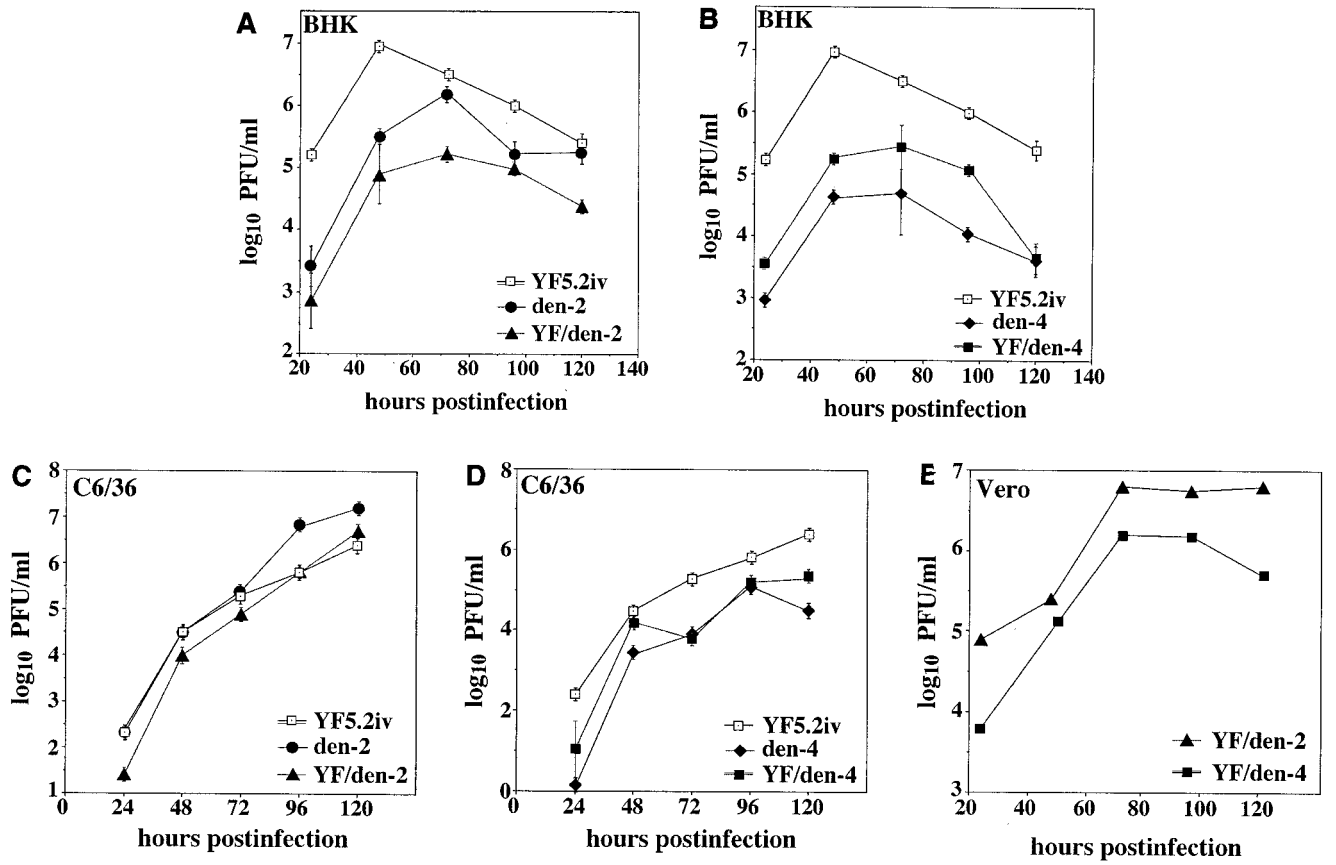

FIG. 4. Growth curves of YF5.2iv, dengue-2 (den-2), and dengue-4 (den-4) viruses and YFV/dengue virus (YF-den) chimeras in cell culture. Cells were infected, and virus yields were measured as described in Materials and Methods. (A and B) Viruses were grown on BHK-21 cells at 37°C. (C and D) Viruses were grown on C6/36 cells at 30°C. (E) Viruses were grown on Vero cells at 37°C. The values for the titers are averages of triplicate samples plus or minus the standard deviation, except for Vero cells, which were duplicate samples.

tion specificities of the chimeric viruses resembled those of the parental dengue viruses with respect to both homologous and heterologous dengue virus antibody, suggesting the presence of authentic, specific antibody epitopes on the E proteins of the chimeric viruses.

Growth properties of chimeric viruses. The growth kinetics of the chimeric viruses were examined in BHK and Vero cells at 37°C and in C6/36 cells at 30°C. YF5.2iv virus (derived from the 17D molecular clone) and Vero-passaged dengue-2 (NGC) and dengue-4 viruses were used for comparison. In BHK cells (Fig. 4A and B), the YF5.2iv parent exhibited the most efficient growth, with a peak titer of $\sim 10^7$ PFU/ml 48 h after infection. The dengue-2 virus NGC strain and chimeric YFV/dengue-2 virus exhibited similar replication kinetics but reached different peak titers of $\sim 10^6$ and $\sim 10^5$ PFU/ml, respectively, 72 h postinfection. The growth kinetics of dengue-4 virus and YFV/dengue-4 virus were also delayed compared to those of YF5.2iv, and these viruses also yielded lower peak titers ($10^{4.5}$ and 10^5 PFU/ml at 72 h, respectively) than YF5.2iv. Thus, the growth properties of the chimeric viruses in BHK cells were more similar to those of their homologous dengue viruses than to those of YF5.2iv. In C6/36 cells (Fig. 4C and D), the growth of dengue-2 virus, YFV/dengue-2 virus, and YF5.2iv followed roughly similar kinetics, although dengue-2 virus reached a slightly higher titer by day 5. The same pattern was observed for dengue-4 virus and YFV/dengue-4 virus, with YF5.2iv growing to higher titers than either of these two viruses. The growth kinetics of the chimeric viruses were also compared on Vero cells (Fig. 4E). YFV/dengue-2 virus reached a higher titer than YFV/dengue-4 virus in this cell line (10^7 PFU/ml versus 10^6 PFU/ml, although the maximal titers were reached at the same time (72 h).

Virulence properties in mice. The neurovirulence of the YFV/dengue virus chimeras was initially assessed by i.c. challenge of 4-week-old outbred (ICR) mice with a fixed dose of 10^4 PFU of virus (Fig. 5). None of the 13 mice which received YFV/dengue-2 virus exhibited any morbidity or mortality after infection at this dose over an observation period of 4 weeks. In contrast, 11 of 12 mice which received YFV/dengue-4 virus succumbed to the infection, with an average survival time of 8.8 days. Thus, the neurovirulence of YFV/dengue-2 virus was completely attenuated at the 10^4-PFU dose, whereas YFV/dengue-4 virus was neurovirulent. To further characterize the neurovirulences of the two chimeras, dose-ranging studies were done (Table 2). YF5.2iv virus was tested over a range of 22 to 0.3 PFU and caused 100% mortality with doses as low as 2 PFU. For YFV/dengue-2 virus, i.c. inoculation of up to 10^6 PFU did not result in any mortality in 4-week-old mice. To

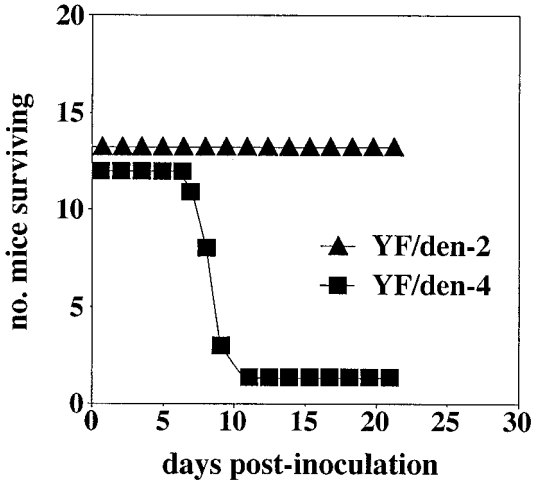

FIG. 5. Mouse neurovirulence assay. Fixed-dose i.c. challenge with 10^4 PFU was carried out in 4-week-old ICR mice. The differences in mortality between the two viruses were significant ($P < 0.005$; Fisher's exact test). YF/den-2 and -4, YF/dengue-2 virus and YF/dengue-4 virus.

determine the levels of virus burdens generated in the brains of mice infected with YFV/dengue-2 virus, 4-week-old mice were inoculated with 80,000 PFU of YFV/dengue-2 virus, and brain-associated virus was measured by plaque assay for 13 days postinfection (Table 3). Virus was detected in mice between 3 and 6 days postinfection but not beyond that point. The titers of virus detected were in the range of 1 to 5 log PFU/g of brain tissue. Only one mouse exhibited any signs of illness (mild to moderate ruffling) during the course of this experiment.

The results of dose ranging with dengue-4 virus revealed that 100% of the mice were susceptible to a dose as low as 3 PFU. The neurovirulence of the YFV/dengue-4 virus was similar to those of the YF5.2iv and dengue-4 viruses, since the majority (80%) of the mice suffered mortality at doses as low as 1 PFU. The average survival times in these experiments were generally similar among the different groups, ranging between 8 and 14 days, depending on the dose of virus. These data indicate that the E protein of the dengue-2 virus strain used in the construction of the YFV/dengue-2 virus chimera led to profound attenuation of neurovirulence associated with the YF5.2iv virus. In contrast, the E protein of the mouse brain-passaged dengue-4 virus conferred a level of neurovirulence on the YFV/dengue-4 virus chimera similar to that of the YF5.2iv virus.

TABLE 2. Neurovirulence testing

Virus	Dose[a]	Mortality		AST[b]
		No.	%	
YF5.2iv	1.34	5/5	100	9.4
	0.34	5/5	100	10.8
	−0.05	1/5	20	14
	−1.52	0/5	0	NA
Dengue-4	2.54	5/5	100	8.2
	1.45	5/5	100	8.2
	0.47	5/5	100	12.8
	−0.5	2/6	33	15
YFV/dengue-4	4.7	6/6	100	8.5
	3.7	5/6	82.5	10
	2.8	6/6	100	9.5
	1.7	6/6	100	11.3
	1.0	5/6	82.5	12
	0	4/5	80	12
YFV/dengue-2	6.2	0/12	0	NA

[a] Doses are in log PFU, based on Vero cell infectivity.
[b] AST, average survival time in days; NA, not applicable.

TABLE 3. Replication of YFV/dengue-2 virus in mouse brain

Day	No. of mice	No. positive	Titer[a]	Range
3	3	1	2.0	NA
5	4	4	4.5	4.3–4.9
6	4	3	2.7	1.0–5
8	4	0	NA	NA
9	4	0	NA	NA
11	4	0	NA	NA
13	3	0	NA	NA

[a] Titer is mean log PFU/gram of brain, based on Vero cell infectivity. NA, not applicable.

Immunogenicity. The capacities of the YFV/dengue virus chimeras to elicit neutralizing-antibody responses were assessed by PRNT on sera of immunized mice. Groups of 3-week-old outbred mice received a single dose of 10^5 PFU of either YFV/dengue-2 virus or YFV/dengue-4 virus or were mock immunized with PBS plus 10% FBS by the intraperitoneal route. None of the mice in either the YFV/dengue-2 virus or YFV/dengue-4 virus group exhibited any morbidity or mortality over the duration of these experiments, which ranged from 4 to 8 weeks, indicating the absence of any neuroinvasiveness associated with these viruses by this route of infection and at this dose. Sera were collected at weekly intervals from individual mice between 4 and 8 weeks postimmunization. Table 4 shows the results of these experiments, with plaque reduction titers shown as the 90% endpoint. Only 4 out of 11 mice immunized with YFV/dengue-2 virus generated detectable antibody. The geometric mean titer (GMT) for YFV/dengue-2 virus recipients was 1:13 (calculated for positive values only), and the values ranged from 1:10 at 6 weeks to a maximum of 1:40 at 8 weeks postimmunization. For YFV/dengue-4 virus, antibody was detectable in 9 out of 11 immunized mice. The GMT was 1:20 at 4 weeks and rose significantly to 1:101 at 6 weeks postimmunization ($P < 0.05$). Collectively, the GMT of all sera from YFV/dengue-4 virus-immunized mice was higher than that of YFV/dengue-2 virus-immunized mice (1:37 versus 1:13; $P < 0.05$ [Mann-Whitney test, two-sided]) despite a shorter time interval between immunization and harvest of sera in the YFV/dengue-4 virus-immunized mice. The use of 70% plaque reduction endpoints for analysis of the data shown in Table 4 slightly increased the percentage of seropositive mice (6 out of 11 dengue-2 virus-immunized mice and 10 out of 11 YFV/dengue-4 virus-immunized mice).

Protection against dengue encephalitis. To determine if immunization of mice with the chimeric viruses was able to induce protection against i.c. challenge with homologous dengue viruses, mice which had received peripheral (intraperitoneal) inoculation with 10^5 PFU of either YFV/dengue-2 virus or YFV/dengue-4 virus were challenged with homologous neuro-

TABLE 4. Postimmunization neutralization titers

Sample no.[a]	Time (wk)[b]	90% PRNT	GMT
YFV/dengue-2 virus			
2.1	6	<1:10	1:13[c]
2.2	6	1:10	
2.3	6	1:10	
2.4	6	1:10	
2.5	6	<1:10	
2.6	7	<1:10	
2.7	7	<1:10	
2.8	7	<1:10	
2.9	7	<1:10	
2.10	8	<1:10	
2.11	8	1:40	
Mock	6	<1:10	
Positive		1:1,000	
YFV/dengue-4 virus			
4.1	4	<1:10	1:20[d]
4.2	4	1:20	
4.3	4	<1:10	
4.4	4	1:20	
4.6	4	1:10	
4.7	4	1:10	
4.8	4	1:80	
4.9	4	1:40	
4.10	6	1:160	1:101[d]; 1:37[c]
4.11	6	1:40	
4.12	6	1:160	
Mock	6	<1:10	
Positive		1:6,000	

[a] Mock and positive are described in Materials and Methods.
[b] Weeks after immunization.
[c] $P < 0.05$ for comparison of YFV/dengue-2 virus and YFV/dengue-4 virus (all samples) groups.
[d] $P < 0.05$ for 4-week versus 6-week samples.

virulent dengue virus (10^6 PFU of mouse brain-passaged dengue-2 virus strain NGC or 10^4 PFU of mouse brain-passaged dengue-4 virus) by i.c. injection. In preliminary experiments, mice 5 to 6 weeks of age were found to be susceptible to these doses (mortality rates, 86% [$n = 7$] for dengue-2 virus and 100% [$n = 12$] for dengue-4 virus). Therefore, to test for protection, mice were immunized at 3 weeks of age and challenge was performed 2.5 weeks later. Control mice were mock immunized with PBS plus 10% FBS. Figure 6 shows the results of these experiments. All mice immunized with YFV/dengue-2 virus resisted challenge and exhibited no signs of illness over an observation period of 3 weeks postchallenge (Fig. 6A). In contrast, 70% of control mice succumbed to challenge, with an average survival time of 9.3 days. The difference between the mortality rates of immunized and mock-immunized mice was significant ($P < 0.05$; Fisher's exact test). Figure 6B shows that all mice immunized with YFV/dengue-4 virus also resisted challenge, in contrast to control mice, 75% of which succumbed to encephalitis with an average survival time of 9.8 days. The difference between the mortality rates of immunized and mock-immunized mice was significant ($P < 0.05$). None of the YFV/dengue-4 virus-immunized mice exhibited any signs of illness postchallenge during an observation period of 3 weeks.

The neutralizing-antibody responses associated with protection were analyzed for some mice which survived challenge in the immunized group, as well as the mock-immunized survivors (Table 5). Antibody was detectable in all such samples. The mock-immunized mice which survived challenge with dengue-2 virus showed postchallenge neutralizing titers which ranged from 1:20 to 1:160 (GMT, 1:60). This value was significantly different from that of immunized but unchallenged mice (1:13 [Table 4]; $P < 0.05$). The titers in YFV/dengue-2 virus-immunized mice which resisted dengue-2 virus challenge ranged from 1:80 to 1:320 (GMT, 1:134) and were significantly different from those of mock-immunized mice ($P < 0.05$). For the YFV/dengue-4 virus experiments, mock-immunized mice surviving challenge with dengue-4 virus exhibited titers ranging from 1:40 to 1:160 (GMT, 1:101), which was significantly different from that of immunized but unchallenged mice (1:20 [Table 4]; $P < 0.05$). YFV/dengue-4 virus-immunized mice exhibited a GMT of dengue-4 virus neutralizing antibody of 1:501 (range, 1:320 to 1:1,280) 4 weeks postchallenge. The

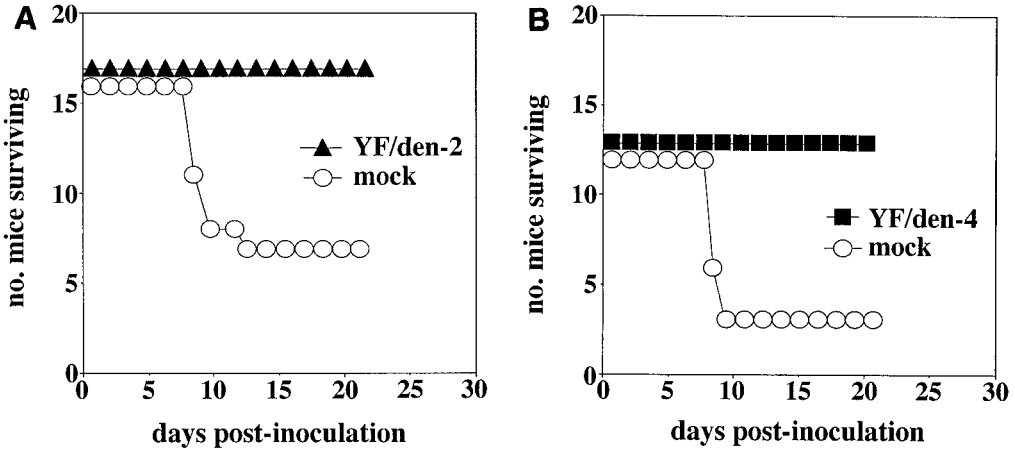

FIG. 6. Protection of mice immunized with YFV/dengue-2 virus (YF/den-2) (A) or YFV/dengue-4 virus (YF/den-4) (B) chimera from challenge with homologous neurovirulent dengue virus. Experiments were conducted as described in Materials and Methods. The percentage of mice succumbing to infection was compared to that of mock-infected mice, and differences in survival were significant for both experiments ($P < 0.05$).

TABLE 5. Postchallenge neutralization titers

Sample no.[a]	Immunized[b]	Time (wk)[c]	90% PRNT	GMT
YFV/dengue-2 virus				
2.11	−	3	1:160	1:60[d,e]
2.12	−	3	1:80	
2.13	−	3	1:80	
2.14	−	3	1:20	
2.15	−	3	1:80	
2.16	−	3	1:160	
2.17	−	3	1:40	
2.18	+	3	1:80	1:134[d,f]
2.19	+	3	1:320	
2.20	+	3	1:80	
2.21	+	3	1:160	
Mock			<1:20	
Positive			1:4,000	
YFV/dengue-4 virus				
4.13	−	3	1:160	1:101[e,g]
4.14	−	3	1:160	
4.15	−	3	1:40	
4.16	+	3	1:320	1:501[f,g]
4.17	+	3	1:320	
4.18	+	3	1:1280	
4.19	+	3	1:320	
4.20	+	3	1:160	
Mock			<1:20	
Positive			1:8,000	

[a] Mock and positive are described in Materials and Methods.
[b] −, not immunized; +, immunized.
[c] Weeks postchallenge.
[d] $P < 0.05$.
[e] $P < 0.05$.
[f] $P < 0.05$.
[g] $P < 0.05$.

difference in GMT compared to mock-immunized mice was significant ($P < 0.05$). The GMTs of mice which survived dengue-4 virus challenge were also significantly different from those of mice which survived dengue-2 virus challenge in the immunized groups (1:501 versus 1:134; $P < 0.05$) but not from those of the mock-immunized groups (1:101 versus 1:60; $P > 0.05$).

Nucleotide sequences of the dengue virus structural protein regions. The nucleotide sequence of the plasmid templates encoding the YFV/dengue-2 virus was identical to that of the dengue virus PUO-218 strain used for its construction (reference 16 and references therein) (data not shown). Dengue-2 virus containing the PUO-218 prM-E region in the background of the NGC strain is highly attenuated for mouse neurovirulence, and a glutamate residue at position 126 of the E protein is a major determinant of this phenotype (16). The high level of attenuation of the YFV/dengue-2 virus chimera was therefore not an unexpected finding and is consistent with many previous reports that the prM-E proteins are principal viral factors governing the virulence and attenuation of flaviviruses. A YFV/dengue-2 virus chimera containing the prM-E region of a South American dengue-2 virus strain closely related to the Southeast Asian genotype was observed to have low mouse neurovirulence (12.5% mortality at a dose of 2.9 $\log_{10}$ PFU in 3-week-old mice) (9). A total of four amino acid substitutions in the prM-E region differentiate this virus from the YFV/dengue-2 virus chimera reported here (prM position 125 [valine versus isoleucine]; E positions 141 [isoleucine versus valine], 484 [valine versus isoleucine], and 494 [glycine versus glutamine]) (data not shown). One or more of these substitutions may account for the small difference in neurovirulence between the two viruses.

Sequence analysis of the prM-E regions of the plasmids encoding the YFV/dengue-4 virus chimera were compared to sequences of other dengue 4 virus strains (the nonneuroadapted strains 814669, 1228, and H241 and the neuroadapted strain H241-N) to gain insight into the molecular determinants involved in its neurovirulent phenotype (Table 6). Compared to that of the H241-N strain (27), the YFV/dengue-4 virus E protein contained three of the four amino acid substitutions that differentiate this neurovirulent virus from its parental H241 strain. These include an isoleucine residue at position 155, a proline residue at position 156, and a leucine residue at position 402, but not an alanine residue at prM position 75. There were 10 positions where the YFV/dengue-4 virus prM-E region differed from that of H241-N. One of these was at prM position 75, and the nine other positions were in the E region. For 8 of these 10 differences, the same amino acid residue was found in YFV/dengue-4 virus and at least two of the three nonneuroadapted viruses 814669, 1228, and H241. At two positions (E_{321} and E_{360}), YFV/dengue-4 virus contained a unique residue.

TABLE 6. Comparison of dengue-4 virus structural proteins

Virus	Amino acid																									
	prM				Envelope																					
	2[e]	52	73	75	23	46	120	140	155	156	163	221	251	265	321	354	357	360	371	373	374	384	402	428	429	455
814669[a]	S	K	T	V	L	T	S	V	T	S	M	T	T	A	T	S	L	N	P	F	G	N	F	L	F	I
1228[b]	H	K	T	V	L	T	L	V	T	S	T	T	T	A	T	S	F	N	P	F	G	D	F	M	F	V
H241[c]	H	E	A	V	S	I	S	I	T	S	T	A	I	T	A	P	F	N	R	L		D	F	L	L	V
H241-N[c]	H	E	A	A	S	I	S	I	I	P	T	A	I	T	A	P	F	N	R	L		D	L	L	L	V
H241-A[d]	H	E	A	V	L	I	S	V	I	P	T	A	T	T	V	S	F	Y	P	F	G	D	L	L	L	V

[a] Genbank no. AF326573.
[b] Reference 20.
[c] Reference 27.
[d] Dengue-4 virus parent of YFV/dengue-4 virus chimera (ATCC no. VR-1257).
[e] Position.

DISCUSSION

The use of YF17D virus as a backbone for the construction of chimeric viruses has been applied to the development of novel vaccines for Japanese encephalitis and dengue viruses (12, 18, 19, 42, 43). The rationale for this approach is based on the efficacy and safety of YF17D vaccine, which has been used for immunization of an estimated 350 million humans. In the study reported here, we characterized the cell culture properties, mouse neurovirulences, and immunogenicities of two YFV/dengue virus chimeras and demonstrated their protective efficacy as experimental vaccines in mice against dengue encephalitis. These chimeric viruses expressed the prM and E proteins of either dengue-2 or dengue-4 virus, and the proteins appeared to be correctly processed, with the E proteins retaining the antigenic properties of the homologous dengue virus. Some cross-reactivity among the E proteins of YFV, dengue-2 virus, dengue-4 virus, YFV/dengue-2 virus, and YFV/dengue-4 virus was observed in immunoprecipitation experiments using polyclonal antisera, most likely involving group-reactive determinants on the E proteins of these viruses. In the PRNT however, higher specificity was observed. The greatest specificity for neutralization of dengue virus and YFV/dengue virus was exhibited by homologous dengue virus antisera. These findings reflect the fact that neutralization assays have the best ability to discriminate among flaviviruses in serologic testing (26) and suggest that authentic dengue virus epitopes on the E proteins of the YFV/dengue virus chimeras participated in their neutralization.

In tests of replication kinetics in cell culture, both YFV/dengue virus chimeras were impaired relative to the YF5.2iv virus in BHK cells, similar to what was observed with the homologous dengue viruses. These effects were less apparent in C6/36 cells, where the peak viral titers of all the viruses differed by ~1.0 log PFU/ml. It is not known at present if this reflects decreased RNA synthesis or less efficient assembly of chimeric-virus particles. Reductions in the growth efficiency of the chimeric viruses may be a consequence of deleterious effects of the dengue virus E proteins on virus spread in cell monolayers, resulting in lower infectivity compared with the parental YFV virus. Effects of the chimeric C-prM-E complex on virus assembly in the cell lines examined in this study could also be a factor. *cis*-acting effects on viral replication involving interactions between dengue virus structural and YFV nonstructural proteins or RNA structures also cannot be excluded on the basis of the present data. In any case, the abilities of the chimeric viruses to replicate to moderate titers in at least three cell lines of different host origins suggest that no unexpected change in the host range properties has emerged in association with creating these viruses.

In studies of mouse neurovirulence, there was a marked difference between the phenotypes of the YFV/dengue virus chimeras. YFV/dengue-2 virus exhibited a nonneurovirulent profile. This attenuation was associated with a moderate virus burden in the brains of young adult mice subjected to i.c. inoculation (peak titers, ~5 log PFU/g), although well below the levels where symptoms of severe encephalitis generally occur (10^7 PFU/g). The prM-E region of this virus is derived from a strain of dengue-2 virus which is highly attenuated for adult mice (16). In contrast to these results, the YFV/dengue-4 virus was highly neurovirulent for mice, with a profile which resembled that of the YF5.2iv virus. This is consistent with data from other studies which implicate the E protein as a virulence factor during dengue-4 virus encephalitis in the mouse model and with neuroadapted dengue-4 virus exhibiting enhanced virulence properties (27). Neither YFV/dengue-2 virus nor YFV/dengue-4 virus exhibited any neuroinvasive properties in our experiments, indicating that no unanticipated virulence phenotypes were exhibited by these viruses in this mouse model. However, unusual tropisms in the peripheral tissues of these mice could exist and cannot be excluded on the basis of the present data. These results suggest that the properties of a chimeric virus utilizing the YF17D backbone can vary depending on the strain of virus used for the derivation of the prM-E proteins. This is most noticeable in tests of neurovirulence in mice, but an effect is also present in terms of immunogenicity (see below). The E protein of mouse-adapted dengue-4 virus may enhance the growth of the YFV/dengue-4 virus chimera in peripheral and neural tissues of mice, leading to a higher virus burden and induction of a stronger host immune response. Although comparison with non-mouse-adapted dengue-4 viruses is required to substantiate this conclusion, an increase in infectivity and immunogenicity as a result of passage in mice has been observed for strains of YFV (14). It is also conceivable that variations in the virulences and immunogenicities of the chimeric viruses could result from defects in assembly of the structural proteins, which might affect infectivity in mice. However, preservation of the native antigenic structure of the dengue virus E proteins in these viruses, as demonstrated by integrity of homologous neutralization epitopes (Table 1) and reactivity with serotype-specific monoclonal antibodies (Fig. 3), suggests that the native forms of the E proteins are present.

Sequence analysis of the plasmids encoding the YFV/dengue-2 virus through the prM-E region confirmed that it represents the dengue virus PUO-218 strain. Based on comparison with the sequences of the attenuated YFV/dengue-2 44/S virus (9) and the attenuated dengue-2 MON310 virus (16), the data are consistent with the presence of glutamate at residue 126 as a principal attenuating determinant of the dengue-2 virus E protein. Genetic studies of a neurovirulent mouse brain-passaged strain of the dengue-2 virus NGC strain and its nonneurovirulent parent (6) revealed that mutations at six amino acid positions in the prM-E region were selected by passage in mouse brain; however, only two residues (lysine at position 126 and aspartic acid at residue 71) were implicated in neurovirulence. It is notable that both the YFV/dengue-2 virus chimera reported here and that reported in reference 9 contain the same six residues found in the nonneuroadapted NGC strain at these sites. Collectively, these various data suggest that substitutions at multiple positions in the E protein occur during adaptation of dengue-2 virus to the mouse brain but that only substitutions at positions 126 and possibly 71 of the E protein affect neurovirulence. In our studies, attenuation of YFV/dengue virus occurred in the background of the YF17D virus, which has a moderate to high intrinsic level of neurovirulence for adult mice. This indicates that the E protein of the dengue-2 PUO-218 virus confers a strong attenuating effect in the context of the YFV/dengue-2 virus chimera.

The neurovirulence of the YFV/dengue-4 virus is consistent with the presence of determinants in the E protein that are

associated with mouse brain adaptation of dengue-4 virus. These include substitution of isoleucine for threonine at position 155, which abolishes the conserved flavivirus N-linked glycosylation site; proline for serine at position 156; and leucine for phenylalanine at position 402 (27). Only positions 155 and 402 have been implicated in the neurovirulence of dengue-4 virus, based on studies of the neuroadapted H241-N virus, and the significance of the P_{156} substitution is unclear (27). Consistent with these findings, the E protein of YFV/dengue-4 virus and parental dengue-4 virus appeared to lack the conserved N-linked glycan (Fig. 2B). The neurovirulent YFV/dengue-4 virus also differed from H241-N at two other positions in the E protein not shared with nonneuroadapted dengue-4 viruses (Table 6). These clonal differences may represent residues selected independently during mouse brain passage, but their significance for the neurovirulent phenotype is not known. The neurovirulence of the YFV/dengue-4 virus for 4-week-old mice was similar to that observed with YF5.2iv in terms of the minimal dose required to cause lethal encephalitis and average survival times. Thus, the E protein of dengue virus, during the process of neuroadaptation, may acquire functional properties which render it similar to that of YF17D virus. Despite this similarity and identification of the candidate genetic determinants for the neurovirulence properties of these viruses, the underlying mechanisms responsible for the phenotype remain undefined. Based on the TBE model for the flavivirus E protein, substitutions at the relevant amino acid positions (155 and 402) may affect the transition to the fusion-active form of the protein, which requires conformational changes in domains I and II, as well as the function of the stem anchor region (1).

Immunization of outbred mice with the YFV/dengue virus chimeras was sufficient to generate detectable levels of neutralizing antibodies to the homologous dengue virus strains. The titers produced were generally of low magnitude, reflecting the relatively poor immunogenicity of YFV and dengue viruses in mice compared to that in humans, where immunization with YF17D elicits virus-specific antibody within several days (38, 57). This presumably reflects adaptation of the more recently evolved YFV and dengue flaviviruses to growth in primate cells (41). In accordance with results obtained using another YFV/dengue-2 virus chimera (56), our study confirms that neutralizing antibodies can be elicited by single-dose inoculation with these viruses, provided sufficient time is allowed for development of a response, and the multiple doses used in other studies (9) may not be required. In fact, as suggested by the former study (56), lower doses of virus may actually result in better immunogenicity, although this remains to be tested. We observed detectable neutralization activity as early as 4 weeks postimmunization, and this increased for at least 1 month, reaching levels above background in approximately half of the immunized mice. All immunized mice which resisted challenge with homologous dengue virus exhibited increases in mean postchallenge titers greater than those observed with mock-immunized mice. This suggests that immunization with the chimeric viruses led to priming of antibody responses, resulting in anamnestic responses in association with protection of many of the challenged mice. Since some of the mock-immunized mice survived challenge, other factors governing resistance to the neurovirulence of dengue viruses in outbred mice of this age also appear to have been operating.

Single-dose immunization of young adult mice with 10^5 PFU of YFV/dengue-2 virus or YFV/dengue-4 virus followed by challenge with homologous dengue virus was sufficient for the chimeras to elicit high-level protective immunity against dengue encephalitis, indicating efficacy as experimental vaccines in the mouse model. Although the value of this model for assessment of candidate dengue virus vaccines for human testing is very limited, the data reported here do not alter the conclusions reached so far on the safety and effectiveness of the YFV/dengue-2 virus serotype and other serotypes which have been tested in nonhuman primate experiments (19–20). It is generally accepted that effective vaccines for the dengue viruses must induce strong neutralizing responses against all four serotypes for a sustained period of time in order to lower the risk of complicated secondary infections associated with ADE. The prechallenge neutralizing-antibody responses observed in our experiments did not appear to be transient responses, and the postchallenge antibody responses correlated with solid protection against disease. The relationship of the findings in this mouse model to the capacity of the experimental vaccines to induce immunologic memory in humans cannot easily be predicted, and their value for this purpose will require considerable investigation.

Notably, the efficacy of this chimeric-virus approach was achieved even with the highly attenuated YFV/dengue-2 virus. In contrast, YFV/dengue-4 virus also behaved as an effective experimental vaccine, but its higher neurovirulence compared with YFV/dengue-2 virus is clearly an undesirable attribute for vaccine development. However, genetic engineering of the YFV/dengue-4 virus E protein to reduce its neurovirulence could be accomplished to eliminate the neurovirulent phenotype. Based on comparisons with other dengue-4 virus sequences, residues 155 and 402 would be most likely to harbor the neurovirulence determinants. Taken together, the various data reported here provide further support for the use of the YFV/dengue virus chimeras as an approach to the development of a live attenuated viral vaccine against the dengue viruses.

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