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Construction of Intertypic Chimeric Dengue Viruses Exhibiting Type 3 Antigenicity and Neurovirulence for Mice

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There are four dengue virus serotypes (DEN1 to -4), each of which causes major epidemics in tropical or subtropical areas. The current strategy for dengue virus immunization favors the use of a tetravalent vaccine preparation. We have previously employed full-length DEN4 cDNA to construct a viable intertypic dengue virus type 1 or type 2 chimera that contained the C-PreM-E or only the PreM-E genes of DEN1 or DEN2 substituting for the corresponding genes of DEN4. This success implied that it might be possible to create mutants of all four dengue virus serotypes for evaluation as candidate vaccines. In this study, we constructed DEN3-DEN4 chimeras that contained DEN3 C-PreM-E genes and expressed DEN3 antigenic specificity. Unlike our previous successes in cloning DEN1 or DEN2 chimeric cDNA, we were not able to clone the DEN3 C-PreM-E genes directly in the 5 intermediate vector or in the full-length chimeric DEN3-DEN4 plasmid in *Escherichia coli*. Nevertheless, a full-length DNA template of DEN3-DEN4 that could be used for transcription of infectious RNAs was prepared by in vitro ligation. Progeny virus recovered from RNA-transfected C6/36 mosquito cells exhibited DEN3 antigenic specificity as determined by a reaction with monoclonal antibodies. Gel electrophoresis of virus-infected cell lysates yielded the predicted viral protein pattern, i.e., DEN3 C, PreM, and E and DEN4 nonstructural proteins. Two amino acid substitutions, Thr-435→Leu and Glu-406→Lys, which are analogous to mutations that, respectively, confer mouse neurovirulence on DEN4 and DEN2, were introduced into DEN3 E. A mutant chimera containing the Thr-435→Leu substitution, which ablates the potential glycosylation site sequence, produced an E protein identical in size to that of wild-type DEN3 E, indicating that the glycosylation site is normally not used. Intracerebral inoculation of suckling mice revealed that the mutant chimera containing the Glu-406→Lys substitution was neurovirulent, whereas its wild-type counterpart or parent DEN3 was not.

Dengue viruses of the *Flaviviridae* family are transmitted to humans principally by two mosquito species, i.e., *Aedes albopictus* and *Aedes aegypti*, and are responsible for dengue outbreaks and epidemics throughout the subtropical and tropical regions. Dengue disease varies in severity, ranging from mild febrile illness to life-threatening dengue hemorrhagic fever and dengue shock syndrome. Worldwide, the dengue viruses cause more disease in humans than any of the other arthropod-borne flaviviruses. The four dengue virus serotypes (DEN1 to -4) are distinguishable by viral neutralization with type-specific polyclonal antibodies or by a binding assay with type-specific monoclonal antibodies. Available evidence indicates that dengue virus infection elicits a resistance against the same dengue virus serotype (homotypic immunity) that is lifelong. On the other hand, immunity against other dengue virus serotypes (heterotypic immunity) is brief, lasting only 6 to 9 months (23). Sequential infection with three different dengue virus serotypes has been reported (18). Because severe dengue is often associated with secondary dengue virus infection (5), it has been suggested that a subneutralizing level of antibodies induced previously during a primary dengue virus infection can form a complex with the infecting dengue virus and thereby facilitate viral entry and subsequent replication in susceptible cells, such as macrophages. It has been proposed that enhancement of viral replication is the immunopathological basis for potentiation of disease leading to dengue hemorrhagic fever or dengue shock syndrome. However, others have presented evidence indicating that dengue hemorrhagic fever and dengue shock syndrome also can occur during the primary dengue virus infection, and in these instances, severe disease was a consequence of infection with dengue viruses of increased virulence that emerged during the late epidemic period (22). The current strategy of dengue virus immunization favors the use of a tetravalent vaccine preparation.

DEN3 was initially isolated and determined to be a distinct virus serotype in 1960 (6). Since that time, numerous isolates of DEN3 have been recovered from dengue outbreaks in Southeast Asia, the Asian Pacific, Africa, and the Caribbean (15). DEN3 has also cocirculated with other dengue virus serotypes during major dengue endemics. Several attempts to develop a live attenuated DEN3 vaccine have been made. In one of the studies, mutants of DEN3 CH53489 that exhibited a small-plaque morphology were selected, but these mutants were not satisfactorily attenuated (9). Serial passage of the same DEN3 strain in primary dog kidney cells was also employed to attenuate its virulence. Viruses at different primary dog kidney cell passages were shown either to be overattenuated or to remain virulent for humans (7). Construction of chimeric viruses with DEN3 antigenic specificity was initiated as an alternative approach to the development of a DEN3 vaccine, because recent studies have shown that construction of a viable chimeric dengue virus bearing genes from two distinct serotypes can bring about attenuation (2, 3). In addition, the ease of introducing strategic mutations into dengue virus genome cDNA and recovering viable mutants has made it possible to produce a variety of mutants that were otherwise not attainable in the past by means of chemical mutagenesis or a variety of selection procedures. In this study, we describe the construction and characterization of DEN3 chimeras that contain DEN3 C-PreM-E structure genes substituting for the DEN4 genes in the DEN4 genome. The DEN3 chimeric cDNA was also used to introduce

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mutations in DEN3 E similar to those previously found in the E gene of mouse-neurovirulent DEN2 and DEN4 mutants. One of the mutant DEN3 chimeras proved to be neurovirulent for mice.

Construction of chimeric DEN3-DEN4 cDNA. DEN3 strain CH53489, kindly supplied by D. Dubois and K. Eckels (Walter Reed Army Institute of Research, Washington, D.C.), was chosen for the construction of intertypic chimeric dengue viruses. The virus was amplified once in C6/36 cells, and the seed virus was used to prepare virion RNA (vRNA) for cDNA cloning. The DEN3 cDNA cloning procedure was similar to that established earlier for the construction of intertypic dengue virus chimeras with type 1 or type 2 antigenicity specificity (2). Briefly, two T_{150} flasks of C6/36 cells were infected with DEN3 CH53489 seed virus at a multiplicity of infection of 0.5 PFU. Culture fluid was collected 7 days after infection, and virions were harvested by centrifugation at 48,000 rpm (Beckman Ti50 rotor) for 2 h. The virion pellet was suspended in STE buffer (0.01 M NaCl, 0.01 M Tris [pH 8.0], 0.001 M EDTA) and treated with 0.5% sodium dodecyl sulfate (SDS) and 200 μg of proteinase K per ml prior to extraction of vRNA with phenol-chloroform. Reverse transcription of vRNA was primed by a complementary sequence of DEN3 at nucleotides (nts) 2401 to 2426, near the 3′ end of the E gene. The cDNA product was used as the template to prepare double-stranded cDNA of the DEN3 C-PreM-E genes in a PCR primed with oligonucleotide D101 containing a flanking *Bgl*II site (2) and oligonucleotide D708 (5′ CGCAATACATGAAAATGACAT GGAAGTGTTTCTCGAGTTCAACCCTATCCA), DEN3 nts 2321 to 2371, carrying three silent nucleotide changes to create an *Xho*I site as underlined. The nucleotide sequence coding for the C-PreM-E polyprotein of DEN3 strain CH53489 and the complete polyprotein sequence of DEN3 strain H87 were determined recently (10, 20). The C-PreM-E DNA fragment contained a *Bgl*II site (nt 86) preceding the first ATG codon (nts 95 to 97), an internal *Xho*I site at nt 1007, and another *Xho*I site (nt 2335) that was introduced near the end of the E gene. The *Bgl*II-*Xho*I fragment (nts 86 to 1006) was first inserted into the p5′-2 intermediate cloning vector (2). In the next cloning step, the *Xho*I fragment (nts 1007 to 2335) was added. Surprisingly, insertion of the *Xho*I fragment to generate the complete DEN3 C-PreM-E cDNA in the p5′-2 vector was not successful. Each of 68 transformants isolated contained the *Xho*I DNA insert in the reverse orientation. In addition, our attempts to insert the *Xho*I fragment to generate a full-length DEN3-DEN4 chimeric plasmid were not successful. Difficulties in constructing a stably cloned full-length cDNA of yellow fever virus (21) or Japanese encephalitis virus (27) have been reported. We then altered our strategy and employed in vitro ligation to construct a full-length template for transcription. To accomplish this, the DEN3 sequence between the *Nsi*I and *Bst*EII sites (DEN3 nts 1111 to 1999) within the DEN3 *Xho*I-*Xho*I fragment (nts 1007 to 2335) was replaced with a DEN4 surrogate sequence between *Bst*EII and *Nsi*I (DEN4 nts 1438 to 1720) (29). This chimeric DEN3-DEN4 DNA fragment was cloned in the desired orientation and remained stable to create a p5′-2 intermediate vector. Subsequently, the remaining 3′ DEN4 nonstructural protein gene sequence was added and cloned as a stable plasmid (14). In the last step of full-length chimeric DNA construction, the DEN4 surrogate sequence was excised and replaced with the missing DEN3 sequence between *Bst*EII and *Nsi*I by ligation without further cloning in *Escherichia coli*. The product in the ligation mixture was then linearized with *Asp*718 and used as the template for transcription with SP6 polymerase.

Chimeric DEN3-DEN4 cDNA mutants. Studies of the mouse-neurovirulent mutant of DEN4 H241 identified two amino acid positions in E as the sites for mutations that lead to acquisition of mouse neurovirulence (13). One of these neurovirulence mutations involves the substitution of Ile for Thr-434 in the DEN4 E. This mutation abolishes the glycosylation site sequence (13). A similar study of the mouse-neurovirulent mutant of DEN2 NGC identified an amino acid substitution at the site in E (Lys for Glu-406) as being most likely responsible for mouse neurovirulence (2). Comparative sequence analysis indicated that the glycosylation site at Asn-433 and the Glu-406 residue represented the corresponding positions in the DEN3 E sequence. We wished to determine whether a DEN3 chimera containing an amino acid substitution in either site in DEN3 E would specify mouse neurovirulence. For this purpose, a single amino acid substitution was introduced at position 406 or 435 of DEN3 E by site-directed mutagenesis. The first mutant contained a substitution of Leu (triplet CTC) for Thr-435 (triplet ACG) of DEN3 E (corresponding to Thr-434 of DEN4 E) which ablated the second potential glycosylation site, and the mutant chimeric cDNA was designated DEN3(T-435→L)-DEN4. This mutation created a *Sac*I site which was used for the subsequent sequence verification of progeny virus. The second mutant contained a substitution of Lys (triplet AAA) for Glu-406 (triplet GAG), and the chimeric cDNA was designated DEN3(E-406→K)-DEN4.

Recovery of chimeric viruses. Confluent C6/36 cells were transfected with the RNA transcripts prepared from wild-type chimeric DEN3-DEN4 cDNA or one of the two mutant chimeric DNA constructs essentially as described previously (2, 14). Transfected cells were monitored by an indirect immunofluorescence assay to detect the presence of DEN3 antigens as evidence for the recovery of progeny chimeric virus. Seven days after transfection, approximately 5 to 20% of the cells were positive by this assay, and at 2 weeks, most cells (80 to 100%) were positive for dengue virus antigens. The fluid medium harvested from each transfection after 2 weeks had a titer of $\sim 10^6$ PFU/ml as determined by a plaque assay of C6/36 cells (1). The plaque assay also revealed that the wild-type DEN3-DEN4 chimeric virus and each of its derived mutant chimeras produced plaques with similar morphologies and that were homogeneous in size. These observations indicated that the RNA infectivity and the growth rates of the mutant constructs containing single amino acid substitutions in C6/36 cells appeared to be similar to those of the parental DEN3-DEN4 chimeric virus. To verify that progeny virus contained the target mutation, vRNA from each mutant chimera was prepared by phenol-chloroform extraction and ethanol precipitation. The vRNA preparation was reverse-transcribed by random priming. The cDNA product was used as the template for PCR with oligonucleotide D101 and oligonucleotide D958 (5′ CA GAAACAGAGTGATTCCTCC, complementary to DEN4 nts 2382 to 2402) (17) as primers. The PCR DNA product of chimera DEN3(E-406→K)-DEN4 was sequenced across the mutation site with oligonucleotide D855 (DEN3 nts 1140 to 1159, 5′ CAACCGACTCAAGATGTCCC). The PCR product of mutant DEN3(T-435→L)-DEN4 was digested with *Sac*I to confirm the presence of the mutated sequence. Figure 1 shows the result of indirect immunofluorescence assay performed to confirm the serotypic specificity of the progeny DEN3 chimera. $LLC\text{-}MK_2$ cells infected with chimera DEN3-DEN4 showed a strong reaction with DEN3-specific monoclonal antibody 5D4 but not with monoclonal antibody 8C2 (DEN1 specific), 3H5 (DEN2 specific), or 1H10 (DEN4 specific). In the control experiment, the latter three antibodies reacted specifically with cells infected with chimera DEN1-DEN4, chimera DEN2-DEN4, and DEN4, respectively.

Analysis of viral proteins. The wild-type DEN3-DEN4 chimeric virus and its two derived mutant chimeras were initially characterized by analysis of their viral proteins. C6/36 cells

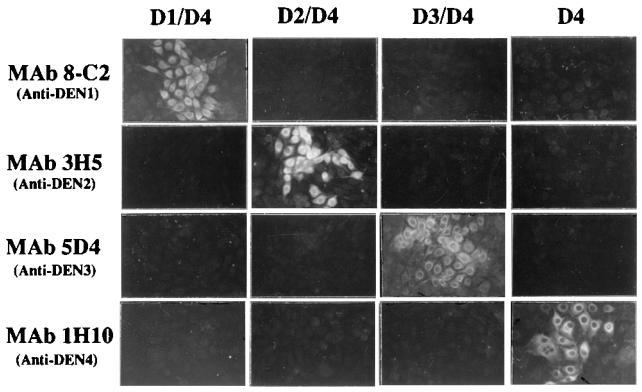

FIG. 1. Immunofluorescence staining of chimeric dengue virus-infected LLC-MK_2 cells. The serotype of the recovered dengue virus chimera DEN3-DEN4 (D3/D4) was determined by indirect immunofluorescence staining. LLC-MK_2 cells infected with other dengue virus serotypes, i.e., DEN1-DEN4 (D1/D4), DEN2-DEN4 (D2/D4), and DEN4 (D4) [recovered from DEN4 cDNA clone 2A(*Xho*I)] were used as controls. Dengue virion serotype-specific monoclonal antibodies (MAb) were used at 1:20 dilutions, and fluorescein-conjugated anti-mouse antibody was used at a 1:100 dilution. Dengue virus serotype-specific monoclonal antibodies including 8C2 (DEN1 specific), 3H5 (DEN2 specific), 5D4 (DEN3 specific), and 1H10 (DEN4 specific) were kindly provided by K. Eckels and J. R. Putnak (Walter Reed Army Institute of Research, Washington, D.C.).

were infected with one of the chimeras, parental DEN3, or DEN4 and labeled with [^{35}S]methionine. As can be seen from Fig. 2A, all three chimeric viruses produced PreM (molecular mass, 20 kDa), E (molecular mass, 55 to 60 kDa), NS1 (molecular mass, 42 to 45 kDa), and NS3 (molecular mass, 71 kDa), which were identified by their migration rates. Also, DEN3 HMAF precipitated more E than did DEN4 HMAF from the lysate of chimera-infected cells. This finding was consistent with the DEN3 specificity of the E product, although DEN4 HMAF was also reactive with chimeric virus E. DEN4 HMAF precipitated more NS1 or NS3 than did DEN3 HMAF, as was expected. DEN3 and DEN4 HMAF detected PreM equally well (Fig. 2A). Figure 2B shows that the E proteins produced by these chimeras migrated to the same position as the parental DEN3 E, whereas the DEN4 E migrated slightly faster. DEN4 PreM also migrated slightly faster than DEN3 or chimeric virus PreM (Fig. 2B). These observations confirmed the identity of DEN3 E or PreM produced by these chimeras.

Treatment of the radiolabeled precipitates with endoglycosidase F was performed to analyze the glycans of the E, NS1, and PreM proteins. The E glycoproteins of all four dengue virus serotypes contain two potential glycosylation sites at corresponding positions (i.e., at Asn-347 and Asn-433 of the DEN4 sequence) upstream of the C-terminal hydrophobic region. Analysis of the glycosylation patterns of DEN4 and its derived mutants indicates that both sites were glycosylated, judging by the gel electrophoresis mobility of E following endoglycosidase F digestion (13). The E protein produced by chimeric DEN3(T-435→L)-DEN4, which contained a mutation in the E glycosylation site sequence, migrated at the same rate as the E protein of parental DEN3 or its derived chimeric virus (Fig. 3). Following endoglycosidase F treatment, the molecular size of the E proteins was reduced, but no discernible difference in the migration rates of the E protein of mutant DEN3(T-435→L)-DEN4 and the E protein of parental DEN3 or its derived DEN3-DEN4 chimera was found. Thus, a mutation created to abolish the second potential glycosylation site had no effect on glycosylation of E. This finding suggests that only the first glycosylation site at Asn-347 of DEN3 E con-

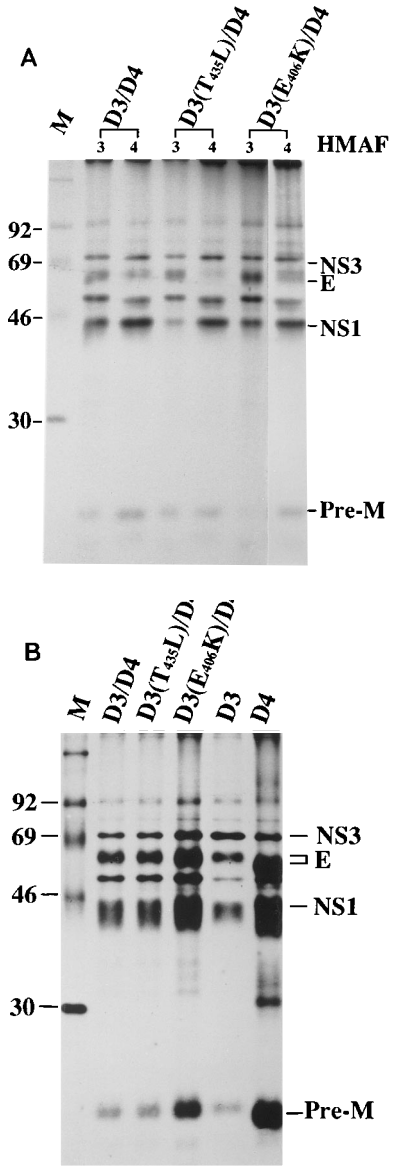

FIG. 2. Analysis on polyacrylamide gels of viral proteins produced by parental and chimeric viruses. Subconfluent C6/36 cells in a T_{25} culture flask were infected with the progeny chimeric virus or the parental DEN3 or DEN4 at multiplicity of infection of 0.5. Four days after infection, cells were labeled with [^{35}S]methionine (100 μCi/ml; specific activity, 600 Ci/mmol) in 2 ml of methionine-free Eagle's minimum essential medium for 4 h and were then lysed in 2 ml of RIPA buffer [1% deoxycholate, 1% Triton X-100, 0.3 M NaCl, 0.1% SDS, 0.1 M Tris (pH 7.5), and 1 mM phenylmethylsulfonyl fluoride]. (A) Aliquots of [^{35}S]methionine-labeled lysates of C6/36 cells infected with chimeric viruses DEN3-DEN4 (D3/D4), DEN3(T-435→L)-DEN4 [D3(T_{435}L)/D4] and DEN3(E-406→K)-DEN4 [D3(E_{406}K)/D4] were immunoprecipitated with DEN3 or DEN4 HMAF (HMAF 3 and HMAF 4, purchased from the American Type Culture Collection, Rockville, Md.), and the precipitates were collected with Pansorbin beads (Calbiochem, La Jolla, Calif.) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The positions of the virus E, PreM, NS1, and NS3 proteins are indicated at the right side. Molecular size markers (in kilodaltons) are shown in lane M. A lane marked with a 3 or a 4 indicates that the lysate was precipitated with HMAF 3 or HMAF 4, respectively. (B) Lysates of C6/36 cells infected with a chimeric virus, parental DEN3, or parental DEN4 virus were immunoprecipitated with a mixture of HMAF 3 and HMAF 4, and the precipitates were analyzed as described previously in this legend.

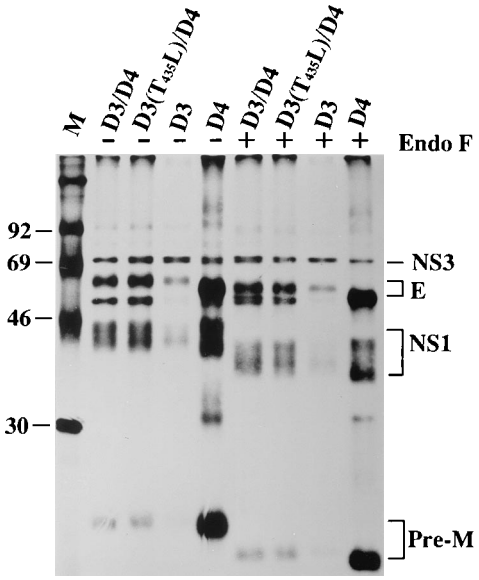

FIG. 3. Endoglycosidase sensitivity of DEN3 E glycoproteins. Radiolabeled lysates of C6/36 cells infected with a chimeric virus or parental DEN3 or DEN4 virus were precipitated with a mixture of DEN3 and DEN4 HMAF. The immune precipitates were digested with endoglycosidase F (Endo F) (+) prior to analysis by SDS-PAGE. Mock digestion (−) served as the control. The locations of dengue virus proteins and the molecular weight markers (lane M) are indicated. Abbreviations are the same as those in the legend to Fig. 2.

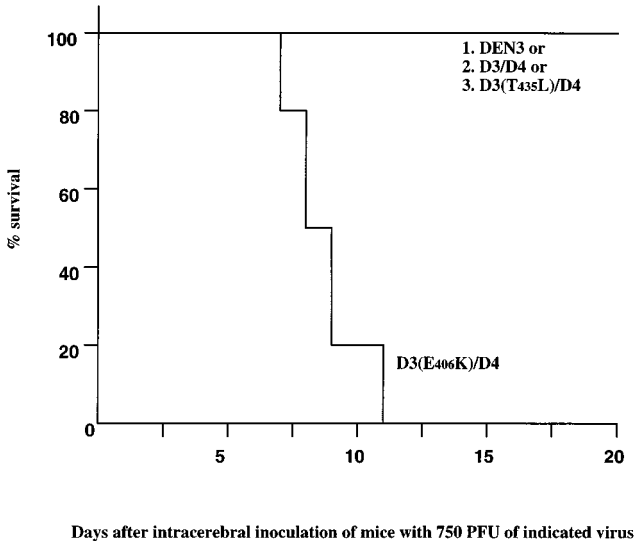

FIG. 4. Mouse neurovirulence of DEN3 and its derived chimeric viruses. Three-day-old outbred Swiss Webster mice in groups of 10 were inoculated intracerebrally with 750 PFU of parental DEN3 or chimeric virus (DEN3-DEN4 [D3/D4]), DEN3(T-435→L)-DEN4 [D3(T_{435}L)/D4], or DEN3(E-406→K)-DEN4 [D3(E_{406}K)/D4]. The latter two chimeras contain a single amino acid change in E as indicated. Inoculated mice were observed daily for 20 days for signs of paralysis and death.

tained the glycan and that the second potential glycosylation site at Asn-433 in DEN3 E was not utilized. Interestingly, the E protein of DEN2 virus is also glycosylated at Asn-347, while the other potential glycosylation site (Asn-433) is not used (12). This indicates that although the overall sequences among dengue virus E proteins are similar, there appear to be sufficient differences in the protein foldings that determine the accessibility of the glycosylation site sequence. The number of potential glycosylation sites in E varies among members of the *Flavivirus* genus. For example, the E protein of Japanese encephalitis virus of the West Nile flavivirus group contains only one glycosylation site, whereas none is present in E of West Nile virus or its related Kunjin virus. The nonglycosylated E proteins of the latter two viruses are functional and immunogenic. It has been shown that deglycosylated flavivirus remains infectious following the removal of the glycans by treatment with endoglycosidase (28). The glycosylation pattern of flavivirus E is also dependent on the cell type or culture conditions. For example, a difference in the gel migrations of the E protein of DEN2 virus grown in Vero cells and the E protein of DEN2 grown in C6/36 cells was also previously observed (26).

Mouse neurovirulence. Intracerebral inoculation of 3-day-old outbred Swiss Webster mice was performed to quantitate the neurovirulence of parental DEN3 and the three DEN3-DEN4 chimeras. In the first experiment, mice in groups of 10 were inoculated with 750 PFU of virus diluted in 25 μl of Eagle's minimum essential medium containing 0.25% human serum albumin. The survival distribution analysis (Fig. 4) showed that all mice inoculated with parental DEN3, DEN3-DEN4, or the DEN3(T-435→L)-DEN4 chimera survived during the 20-day observation period. On the other hand, each of the mice inoculated with the same dose of the DEN3(E-406→K)-DEN4 chimera developed symptoms of encephalitis and eventually died by day 11. Thus, the mutant chimera DEN3(E-406→K)-DEN4 acquired mouse neurovirulence, a property not exhibited by parental DEN3 or chimeric DEN3-DEN4 or DEN3(T-435→L)-DEN4. In the second experiment (Table 1), neurovirulence was evaluated over a range of virus titers. The 50% lethal dose (LD_{50}) of mutant DEN3(E-406→K)-DEN4 was 79 PFU. In contrast, parental DEN3 or its derived chimera was not neurovirulent at a virus dose of 1,500 PFU. This finding indicates that the substitution E-406→K in E was responsible for the acquisition of DEN3 mouse neurovirulence. Mutations involving charged amino acid changes in E of the parent DEN1 or DEN2 to their respective mutants selected by serial passage in mouse brain have been found. Selection of monoclonal antibody escape mutants from wild-type DEN2 also resulted in a change of charged amino acid residues in E (16). Similarly, a monoclonal antibody escape mutant of Louping ill virus also contained a charged amino acid substitution of Asn for Asp at position 308 in E (amino acids 1 to 496). Interestingly, the escape mutant of Louping ill virus exhibited reduced mouse neurovirulence (11). A compar-

TABLE 1. Mouse LD_{50} analysis of DEN3 strain CH53489 and its derived chimeric viruses[a]

Virus[b]	Dose (PFU)	Mice (survived:tested)	Calculated LD_{50} (PFU)
DEN3	1,500	10:10	>1,500
DEN3-DEN4	1,500	10:10	>1,500
DEN3(T-435→L)-DEN4	1,500	10:10	>1,500
DEN3(E-406→K)-DEN4	750	0:10	79
	150	2:10	
	30	7:10	
	6	9:10	
	1.2	10:10	

[a] Three-day-old outbred Swiss Webster mice were inoculated intracerebrally with each virus at the dose indicated. Inoculated mice were observed for 20 days, and the number of survivors was recorded.

[b] In previous studies, DEN4 exhibited very little mouse neurovirulence; the calculated LD_{50} was >1,000 PFU (13).

ison of the sequences of the prototype and the vaccine strain of Japanese encephalitis virus or yellow fever virus also showed several charged amino acid changes (4, 19). These findings suggest that substitution of charged amino acid residues in E of the wild-type flaviviruses may attenuate neurovirulence.

Serial passage of DEN1 or DEN2 virus in mouse brain was employed earlier to select for mutants that were evaluated for attenuation in humans (8, 23–25). The early studies showed that the dengue virus mouse neurovirulent mutants selected at low to intermediate passage levels (approximately 7 to 25 passages) exhibited significant attenuation for humans. Mouse intracerebral passage, apparently in a concerted manner, also selected for mutants that exhibited increased mouse neurovirulence. This suggests the possibility that the genetic determinants responsible for dengue virus mouse neurovirulence may be directly associated with the mutations that confer attenuation for humans. To test that hypothesis, a series of laboratory-generated mutants of DEN3 or other serotype specificities will be evaluated when an animal model for human dengue disease becomes available.

The successful construction of intertypic chimeric dengue viruses that exhibit type 1, type 2, or type 3 antigenicity offers a novel approach to the development of live dengue virus vaccines. First, chimeric viruses may be attenuated by creating a mixed constellation of dengue virus genes as suggested by ongoing studies with rhesus monkeys (3). In addition, observations made during a previous study indicate that a DEN2-DEN4 chimera containing the C-PreM-E genes of DEN2 replicated less efficiently than its DEN2 parent (2). The reduction of replication efficiency may stem from the chimeric gene constellation which programs the synthesis of the DEN4 nonstructural proteins and the structure proteins of another dengue virus serotype. The combination of these intertypic dengue virus proteins could result in heterologous protein-protein or protein-RNA interactions. Such interactions may be suboptimal for viral replication and thus result in attenuation. Results obtained from a recent study of chimeric DEN1 and DEN2 viruses in primates indicated that the infectivity of these chimeric viruses as measured by days of viremia was reduced compared with that of their dengue virus type 1 or type 2 parent (3). For this reason, the intertypic chimera that expresses (i) DEN1 or DEN2 antigenicity studied earlier or (ii) DEN3 antigenicity as in this study might be satisfactorily attenuated without further modification.

Second, it may be possible to introduce mutations in the shared DEN4 region of these intertypic chimeras that might confer attenuation. These chimeric viruses share approximately 80% of the DEN4 genome sequence that includes the 5′ and 3′ noncoding regions and the genes encoding all nonstructural proteins. Our strategy has been first to engineer DEN4 mutants, preferably those containing deletion mutations in the above-mentioned regions, and as a first step evaluate these DEN4 mutants for alteration of growth properties in cultured cells. Viruses containing mutations that cause restriction of dengue virus replication in cultured cells are selected as candidate vaccines for evaluation in primates because many licensed live-virus vaccines exhibit this phenotype. The candidate vaccine mutants that prove to retain a high level of immunogenicity in primates will be further evaluated in humans. If mutations that confer satisfactory attenuation of DEN4 can be identified, it may be possible to transfer such attenuating mutations to the chimeric genomes of other serotypes. In this manner, construction and evaluation of attenuated chimeric dengue viruses with type 1, type 2, or type 3 serotype specificity might be achieved with only the genetic contribution of DEN4 being used as the site for the introduction of attenuating mutations.

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