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Chimeric Tick-Borne Encephalitis and Dengue Type 4 Viruses: Effects of Mutations on Neurovirulence in Mice

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Two new chimeric flaviviruses were constructed from full-length cDNAs that contained tick-borne encephalitis virus (TBEV) CME or ME structural protein genes and the remaining genes derived from dengue type 4 virus (DEN4). Studies involving mice inoculated intracerebrally with the ME chimeric virus indicated that it retained the neurovirulence of its TBEV parent from which its pre-M and E genes were derived. However, unlike parental TBEV, the chimeric virus did not produce encephalitis when mice were inoculated peripherally, indicating a loss of neuroinvasiveness. In the present study, the ME chimeric virus (vME) was subjected to mutational analysis in an attempt to reduce or ablate neurovirulence measured by direct inoculation of virus into the brain. We identified three distinct mutations that were each associated independently with a significant reduction of mouse neurovirulence of vME. These mutations ablated (i) the TBEV pre-M cleavage site, (ii) the TBEV E glycosylation site, or (iii) the first DEN4 NS1 glycosylation site. In contrast, ablation of the second DEN4 NS1 glycosylation site or the TBE pre-M glycosylation site or amino acid substitution at two positions in the TBEV E protein increased neurovirulence. The only conserved feature of the three attenuated mutants was restriction of virus yield in both simian and mosquito cells. Following parenteral inoculation, these attenuated mutants induced complete resistance in mice to fatal encephalitis caused by the highly neurovirulent vME.

Following the success in constructing full-length dengue type 4 virus (DEN4) cDNA that can be transcribed to produce infectious RNA (15), cDNAs of chimeric tick-borne encephalitis virus/DEN4 (TBEV/DEN4) constructs were prepared, and RNA transcripts of these cDNAs were shown to be infectious for permissive simian cells (20). Two new flaviviruses were recovered that contained TBEV capsid (C), membrane (M), and envelope (E) or ME structural protein genes with the remaining genes derived from DEN4. The chimeric TBE(ME)/DEN4 virus was investigated in more detail, and it was observed that TBE(ME)/DEN4 virus replicated more efficiently than DEN4 in simian cells, reaching a titer 1,000-fold higher than DEN4, but it grew to a titer 100-fold lower compared with DEN4 in mosquito cells. Evaluation of chimeric virus in mice revealed that chimeric TBE(ME)/DEN4 virus caused fatal encephalitis in suckling and adult mice following intracerebral (i.c.) inoculation, whereas adult mice inoculated with DEN4 remained free of disease and few suckling mice developed encephalitis. Thus, the chimeric virus retained the neurovirulence of TBEV from which its pre-M and E genes were derived. In contrast, unlike parental TBEV, TBE(ME)/DEN4 virus was not virulent when adult BALB/c mice were inoculated peripherally, indicating a loss of neuroinvasiveness. Mice inoculated peripherally with TBE(ME)/DEN4 virus were protected against subsequent intraperitoneal (i.p.) challenge with a lethal dose of TBEV.

Success in constructing a viable TBEV/DEN4 chimera that retains the protective antigens of TBEV but lacks the peripheral invasiveness of TBEV provides the basis for pursuing the development of an attenuated TBEV vaccine. However, before this goal can be realized, additional modifications of the chimera must be achieved to ablate neurovirulence for the central nervous system as measured by direct inoculation of virus into the brain. Analysis of amino acid sequences of a variety of flaviviruses indicates that potential N-linked glycosylation sites in the pre-M, E, or NS1 glycoprotein vary in number and in position, although similarities are also found (22, 28). The functional role that the carbohydrate moiety of these glycoproteins plays during viral replication and in the pathogenesis is not known. Studies on a number of other viral systems including ortho- and paramyxoviruses indicate that proteolytic cleavage of the attachment glycoprotein which mediates membrane fusion is essential for viral replication and that there is a strict correlation of cleavage and pathogenesis (4). Flavivirus pre-M also undergoes cleavage during the late stage of viral maturation. There is evidence demonstrating that flavivirus E glycoprotein is capable of mediating membrane fusion at a low pH and that this activity is blocked if cleavage of pre-M to generate M is inhibited (9, 10). Upon addition of acidotropic agents, such as NH_4Cl, to flavivirus-infected cells, viral assembly continues to take place despite the fact that cleavage of pre-M is inhibited. Pre-M-containing virions produced intracellularly are reported to be less infectious than the extracellular M-containing virus particles (23). Engineering defined mutations in the DEN4 or TBEV portion of the chimeric genome should ultimately yield mutants that lack mouse neurovirulence. In this study, several strategic mutations were introduced into the full-length TBE(ME)/DEN4 virus cDNA construct. Six mutant chimeric viruses were recovered from transfected simian cells and analyzed for the effect of these mutations on growth of

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virus in cell culture, mouse neurovirulence, and protective efficacy.

MATERIALS AND METHODS

Construction of plasmids. Restriction enzymes were purchased from GIBCO BRL, Life Technologies, Inc., or New England BioLabs, Inc. A GEMSEQ kit or Sequenase 2.0 kit (United States Biochemical Corp.) was used for DNA sequencing. Oligonucleotides (oligos) encoding amino acid substitutions were synthesized on model 391 PCR-Mate oligosynthesizer (Applied Biosystems, Inc.). All mutant chimeric plasmids were constructed on the background of the pTBE(ME)/DEN4 (20), which contains a unique *Not*I site, SP6 RNA polymerase promoter, the 5′ noncoding region and C gene of DEN4, and the pre-M and E genes of TBEV (from 415 to 2376 nucleotides [nts]), and the remaining sequences that included the NS1 through NS5 genes and 3′ noncoding region were derived from the DEN4 genome immediately preceding the *Asp*718 site. The TBEV genome nucleotide numbers and polyprotein amino acid residues are cited according to their designation in Pletnev et al. (22); DEN4 genome nucleotide and polyprotein amino acid designations are those of Mackow et al. (16) and Zhao et al. (28).

Oligo 239 (5′-GACCGACAAGGACAGTTCCAAATCG GA) (DEN4 sequence between nts 18 and 44) and oligo 437 (5′-GATGGGATCAGCACTACGCGTCTTGTTCTTGATC CTTCTTG) (complementary to TBEV nts 718 to 758; mutated residues are underlined) were used for a polymerase chain reaction (PCR) with *Taq* polymerase (Perkin-Elmer Cetus Corp.), with pTBE(ME)/DEN4 as a template. The PCR product was digested by *Hin*dIII and *Mlu*I at sites present at the ends of this DNA fragment. Oligo 436 (5′-GG ATCAAGAACAAGACGCGTAGTGCTGATCCCATCCC AC) (TBEV nts 724 to 762) and oligo 1451, which is complementary to the DEN4 sequence at nts 2371 to 2418, were used for another PCR product. The product was digested by *Mlu*I and *Xho*I. Both *Hin*dIII-*Mlu*I- and *Mlu*I-*Xho*I-digested PCR products were inserted into the corresponding *Hin*dIII-*Xho*I-digested p5′-2(Δ*Pst*I, *Xho*I, Δ*Hin*dIII) plasmid (20) to generate a plasmid containing a new *Mlu*I site in the pre-M gene and substitution of a Ser-206 residue for Val in the pre-M protein. The 3′ half of the DEN4 genome cDNA was introduced in this mutant DNA plasmid by joining it with the *Bst*BI-*Kpn*I fragment from p2A (15). The final mutant plasmid was designated as pTBE(ME: pre-M, Ser-206→Val)/DEN4. The same strategy was used for construction of a mutant containing two substitutions in pre-M: Arg-204→Val and Ser-206→Val. In this case, oligo 239 and oligo 438 (5′-GATGGGATCAGCACTACGCGTAC TGTTCTTGATCCTTCTTG (complementary to TBEV nts 718 to 758) were used for PCR to produce a DNA fragment with these two mutations. The final construct was designated pTBE(ME: pre-M, Arg-204→Val, Ser-206→Val)/DEN4. Using pairs of oligos (oligo 239 plus oligo 440 [5′-TTTTCTTC CACTGTGAGTCTCTAGAGCAGCGACGTAATCCCCC, complementary to TBEV nts 1407 to 1449] and oligo 439 [5′-GGGGGATTACGTCGCTGCTCTAGAGACTCACAG TGGAAGAAAA, TBEV nts 1407 to 1449]) plus oligo 1451, a new *Xba*I site was generated in the E gene sequence resulting in substitution of Leu for Asn-434 in the potential N-glycosylation site of TBEV E protein. The final plasmid was designated pTBE(ME: E, Asn-434→Leu)/DEN4. To prevent N-linked glycosylation of the pre-M protein, oligo 445 (5′-GCGGCAACCCAGGTGCGTGTCGATCGTGGCA CCTGTGTGATCCTGG [TBEV nts 532 to 577]) and oligo 446 (5′-CCAGGATCACACAGGTGCCACGATCGACACG CACCTGGGTTGCCGC [complementary to TBEV nts 532 to 577]) were used to introduce a new *Pvu*I site in the pre-M gene and generate substitutions Glu-143→Asp and Asn-144→Arg in the pre-M protein. This plasmid was designated pTBE(ME: pre-M, Glu-143→Asp, Asn-144→Arg)/DEN4. Substitution of His-384→Gly was introduced in the E protein with oligo 427 (5′-GCCCCAGCCTCGATCGCTCTGGT CTCTCTTACACAC [complementary to TBEV nts 1237 to 1272]) plus oligo 239 and oligo 431 (5′-GAGAGACCAGA GCGATCGAGGCTGGGGCAACGGGTGTGGATT ATTTGGAAAAGGC [TBEV nts 1245 to 1299]) plus oligo 1451. Two mutations, His-384→Gly and Leu-387→Phe, were introduced into the E protein with oligo 427 plus oligo 239 and oligo 428 (5′-AGAGACCAGAGCGATCGAGGC TGGGGCAACGGGTGTGGATTTTTGGAAAAGGCAG CATTGTG [TBEV nts 1246 to 1308]) plus oligo 1451 for PCR. Final plasmids were designated pTBE(ME; E, His-384→Gly)/DEN4 and pTBE(ME: E, His-384→Gly, Leu-387→Phe)/DEN4.

Two other constructs were prepared which had mutations in the DEN4 NS1 portion of the chimeric pTBE(ME)/DEN4 plasmid. Oligo 442 (5′-GTCCGTCTATTAAAAATGTGC GGATCCTTGCTTCTGGGGTGAA [complementary to DEN4 nts 2790 to 2832]) and oligo 431, which encoded the TBEV sequence from nts 1245 to 1299, were used for PCR, and the DNA product was digested by *Xho*I and *Bam*HI. A second PCR fragment was prepared with oligo 441, which was complementary to oligo 442, and oligo 2634, which was complementary to the DEN4 genome from nts 5090 to 5110; this PCR product was digested by *Bam*HI and *Bst*BI. Both PCR DNA fragments were joined and inserted into *Xho*I-*Bst*BI-digested pTBE(ME)/DEN4, to generate pTBE(ME)/DEN4(NS1, Asn-903→Ile, Ser-904→Arg), which contained mutations resulting in substitutions Asn-903→Ile and Ser-904→Arg in the first potential N-glycosylation site of the NS1 protein. Similarly, mutations leading to substitutions Lys-979→Arg and Asn-980→Ile in the second potential glycosylation site of NS1 protein were introduced by PCR with oligo 444 (5′-TCTCTATCTGCCAAGTCTGGATCCT TGAGCTCTCTATCCA [complementary to DEN4 nts 3021 to 3060]) plus oligo 431 and oligo 2634 plus oligo 443, the complement of oligo 444. The resulting plasmid was designated pTBE(ME)/DEN4(NS1, Lys-979→Arg, Asn-980→Ile). The mutations introduced into each plasmid were verified by DNA sequence analysis.

Isolation and characterization of mutant viruses. In vitro RNA transcription of the plasmid template, transfection of RNA (1 to 2 μg) into permissive simian LLC-MK$_2$ cells, detection of antigen-positive cells with TBEV- or DEN4-specific hyperimmune mouse ascitic fluid (HMAF), immunofluorescence assay, plaque assay, and characterization of growth properties of mutant viruses on LLC-MK$_2$ or mosquito C6/36 cells were done as described previously (2, 5, 15, 20).

Six mutant chimeric TBE(ME)/DEN4 viruses proved to be viable, as indicated in Table 1. One week after transfection, cells from a 25-cm^2 flask were transferred together with 10^6 uninfected cells to a 150-cm^2 flask containing 20 ml of fresh M199 medium supplemented with 10% fetal calf serum. Cells were incubated for an additional 4 to 6 days. Cultures were harvested when 80 to 100% of cells were positive by immunofluorescence assay with DEN4 or TBEV-specific HMAF. In this manner, stocks of virus were prepared after second passage in LLC-MK$_2$ cells.

To verify the genomic structure of mutant TBE(ME)/

TABLE 1. Mutant TBE/DEN4 plasmid constructs, their viability, and designation of recovered virus

Plasmid containing sequences for:	Virus recovered	Designation of virus
Unmutagenized virus		
DEN4	+	DEN4
TBE(ME)/DEN4 chimera	+	vME
TBE(CME)/DEN4 chimera	+	vCME
Mutagenized TBE(ME)/DEN4 chimera		
Pre-M cleavage mutants		
TBE(ME: pre-M, Ser-206→Val)/DEN4	+	vPreM(V_{206})
TBE(ME: pre-M, Arg-204→Val, Ser-206→Val)/DEN4	−	
Pre-M glycosylation site mutant TBE(ME: pre-M, Glu-143→Asp, Asn-144→Arg)/DEN4	+	vPreM(D_{143}, R_{144})
E glycosylation site mutant TBE(ME: E, Asn-434→Leu)/DEN4	+	vE(L_{434})
E mutants		
TBE(ME: E, His-384→Gly)/DEN4	−	
TBE(ME: E, His-384→Gly, Leu-387→Phe)/DEN4	+	vE(G_{384}, F_{387})
NS1 glycosylation site mutants		
TBE(ME)/DEN4(NS1, Asn-903→Ile, Ser-904→Arg)	+	vNS1(I_{903}, R_{904})
TBE(ME)/DEN4(NS1, Lys-979→Arg, Asn-980→Ile)	+	vNS1(R_{979}, I_{980})

DEN4 viruses, we reverse transcribed RNA isolated from mutant-infected LLC-MK_2 cells using oligo 2634. The cDNA was used as the template for PCR with the primer pairs oligo 239 plus oligo 1451 or oligo 431 plus oligo 2634. The PCR DNA products were digested by *Hin*dIII and *Xho*I or *Xho*I and *Bst*BI and cloned in p5′-2(Δ*Pst*I, *Xho*I, Δ*Hin*dIII) or p5′-2 vector (5, 20). Sequence of the mutant genome between TBEV nts 504 and 649 of vPreM(D_{143}, R_{144}), nts 660 and 841 of vPreM(V_{206}), nts 1215 and 1380 of vE(G_{384}, F_{387}), nts 1341 and 1508, 2745 and 2881, or 2993 and 3165 of vE(L_{434}) or DEN4 nts 2744 and 2860 of vNS1(I_{903}, R_{904}) or nts 2998 to 3124 of vNS1(R_{979}, I_{980}) was confirmed by sequencing the cloned DNA fragments.

To analyze proteins produced by the parental chimera (hereafter designated vME) or mutant chimeric vME, vCME, or DEN4, we infected confluent LLC-MK_2 cells in a 25-cm^2 flask at a multiplicity of infection of 0.01 PFU per cell. Six days after infection, cells were incubated with L-[^{35}S]methionine (100 μCi per well, >800 Ci/mmol; Amersham Corp.) in methionine-free Eagle's minimal essential medium for 4 h, rinsed with 1 ml of cold phosphate-buffered saline, and then lysed with 1 ml of RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.5). Lysates were incubated with TBEV- or DEN4-specific HMAF, DEN4 NS1-specific monoclonal antibody, 8E2-2 rabbit TBEV E-specific serum, or rabbit DEN4 pre-M, E, NS3, or NS5-specific serum overnight in RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS). Antibody-antigen complexes were collected with excess Pansorbin beads (Calbiochem), and precipitates were washed three times with 0.5 ml of RIPA buffer containing 2% SDS and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (14).

Mouse neurovirulence of parental and mutant vME. To estimate the pathogenicity of DEN4, chimeric ME or CME virus, or mutants of chimeric vME, 6-week-old female BALB/c mice were inoculated i.c. with 0.1, 1, 10, 100, 1,000, or 10,000 PFU of virus in 0.03 ml of minimal essential medium–0.25% human serum albumin. Mice were observed for 31 days for symptoms of encephalitis or death. To evaluate neuroinvasiveness of DEN4, chimeric ME virus, or its mutants, 6-week-old female mice in groups of eight were inoculated (i) i.c. with 10^3 or 10^4 PFU of DEN4, chimeric vME, or one of its mutants in 0.03 ml diluted as above, or (ii) i.p. or intramuscularly (i.m.) with 10^4, 10^5, 10^6, or 10^7 PFU of virus in 0.10 ml of dilutant. Mice were observed for 28 days. To determine the protective efficacy and immunogenicity of parental and mutant ME viruses, five female adult BALB/c mice were inoculated i.p. or i.m. with 10^4 PFU of virus in 0.1 ml of minimal essential medium or i.c. with 10 PFU of virus in 0.03 ml of minimal essential medium 0.25% human serum albumin. Mice were observed for 21 days, and surviving mice were bled 22 days after inoculation to evaluate antibody response. Surviving mice were challenged i.c. at 25 days with 10^4 PFU of parental ME virus and observed for an additional 4 weeks.

RESULTS

Design of mutant viruses. We previously used DEN4 cDNA as a cloning vector to construct chimeric TBEV/DEN4 viruses containing C–pre-M–E or pre-M–E genes of TBEV and showed that these chimeras were able to replicate in primate-derived LLC-MK_2 and mosquito-derived C6/36 cells (20). Studies of mouse neurovirulence revealed that the chimeric vME was able to induce protective immunity against fatal TBEV peripheral challenge in mice. However, the chimera retained the neurovirulence phenotype of its TBEV parent as assayed by i.c. inoculation. Site-directed mutagenesis with synthetic oligonucleotides was performed to introduce one or more defined mutations in the DEN4 NS1 or TBEV pre-M or E gene in order to produce vME mutants that could be evaluated for the effect of such mutations on mouse neurovirulence. The first set of mutations was designed to ablate potential glycosylation sites in the TBEV pre-M or E or DEN4 NS1 protein. As shown in Table 1, four mutant constructs were prepared: (i) pTBE (ME: pre-M, Glu-143→Asp, Asn-144→Arg)/DEN4, which

contained mutations leading to a substitution Asn-144→Arg in the pre-M glycosylation site Asn-144-Gly-Thr and another substitution of Glu-143→Asp that preceded the glycosylation sequence; (ii) pTBE(ME: E, Asn-434→Leu)/DEN4, which encoded an Asn-434→Leu substitution at the potential glycosylation site of E (Asn-434-Glu-Thr); (iii) pTBE (ME)/DEN4(NS1, Asn-903→Ile, Ser-904→Arg), which contained mutations resulting in substitutions of Ile for Asn-903 and Arg for Ser-904 in the first potential glycosylation site (Asn-903-Ser-Thr) of the DEN4 NS1 protein; and (iv) pTBE (ME)/DEN4(NS1, Lys-979→Arg, Asn-980→Ile), which contained mutations resulting in substitutions of Arg for Lys-979 and Ile for Asn-980 at the second potential glycosylation site (Lys-979-Asn-Gln-Thr) of the DEN4 NS1 protein. Virus was recovered from each of these constructs.

The second type of mutant was designed to yield a noncleavable pre-M. For this purpose, two different plasmid cDNAs were constructed: (i) pTBE(ME: pre-M, Ser-206→Val)/DEN4, which contained a mutation which encoded a substitution of Ser-206→Val, and (ii) pTBE(ME: pre-M, Arg-204→Val, Ser-206→Val)/DEN4, which contained a mutation which resulted in substitution of Arg-204→Val and Ser-206→Val. These substitutions altered the putative pre-M/M cleavage sequence Arg-202-Thr-Arg-Arg/Ser. Only chimeric mutant PreM(V_{206}) which contained the substitution of Val for Ser at residue 206 was viable.

The third type of mutant contained amino acid substitutions in the region of TBEV E protein between amino acids 378 and 391, a region highly conserved among flaviviruses (22). It has been suggested that this region plays a role in acid-catalyzed fusion (9, 10). His at position 384 is unique for members of the TBEV subgroup of viruses (13, 17, 18, 21, 24, 26). Mutation in pTBE(ME: E, His-384→Gly)/DEN4 resulted in substitution of Gly for His-384, thus making this position of the E protein the same as that of the mosquito-borne viruses. In addition, pTBE(ME: E, His-384→Gly, Leu-387→Phe)/DEN4 was constructed to generate two substitutions, His-384 to Gly and Leu-387 to Phe, in the conserved sequence. The latter substitution in the E protein sequence is found in the attenuated vaccine strain of Japanese encephalitis virus (1, 19). Only the RNA transcripts containing the double mutation were capable of producing progeny virus, designated vE(G_{384}, F_{387}) (see Table 1 for designation of virus). Repeated transfection attempts failed to yield a viable chimeric mutant containing the single amino acid substitution at residue 384.

Transfection experiments with pig embryo kidney cells (22) were performed to determine and compare the RNA infectivities of the parent virus and its derived mutant constructs. The result showed that the specific infectivity was 1,200 to 2,000 PFU/μg for vME RNA, 860 to 1,000 PFU/μg for vPreM(V_{206}), and 340 to 1,100 PFU/μg for vE(L_{434}). Thus, under the experimental conditions described in Materials and Methods, the parental and both mutant RNAs yielded similar titers of the respective progeny virus during the initial transfection cycle.

Analysis of proteins in mutant-infected cells. As a first step in the characterization of these mutant chimeras, ^{35}S-labeled lysates of virus-infected LLC-MK_2 cells were immunoprecipitated with DEN4- or TBEV-specific HMAF (Fig. 1). The protein profile of vPreM(V_{206}) and vPreM(D_{143}, R_{144}) (Table 1) was similar to that of parental vME. Because TBEV-specific HMAF or rabbit antiserum failed to precipitate TBEV pre-M or its cleaved M protein, we were not able to detect the modification of pre-M cleavage predicted for the vPreM(V_{206}) mutant or the modification of pre-M glycosylation predicted for the vPreM(D_{143}, R_{144}) mutant. In addition, two amino acid substitutions in the E protein of vE(G_{384}, F_{387}) did not change gel mobility. On the other hand, an alteration of gel migration was readily detected for the NS1 protein of vNS1(I_{903}, R_{904}) or vNS1(R_{979}, I_{980}) as expected for mutation in either of the glycosylation sites. The NS1 product of both mutants migrated faster than the NS1 protein of parental vME. It appears that the NS1 product of vNS1(R_{979}, I_{980}) migrated slightly faster than the NS1 product of vNS1(I_{903}, R_{904}) (Fig. 1 and 2). The NS1 protein of vNS1(I_{903}, R_{904}) or vNS1(R_{979}, I_{980}) was sensitive to digestion by endoglycosidase H (endo H), or endoglycosidase F (endo F) as indicated by a 3- to 5-kDa reduction of estimated molecular mass (M_r) following the treatment (Fig. 2). This indicates that the NS1 protein of the mutant chimeras contained only one of the two carbohydrate side chains normally added to the polypeptide. To examine whether such a glycosylation defect affected the formation of NS1

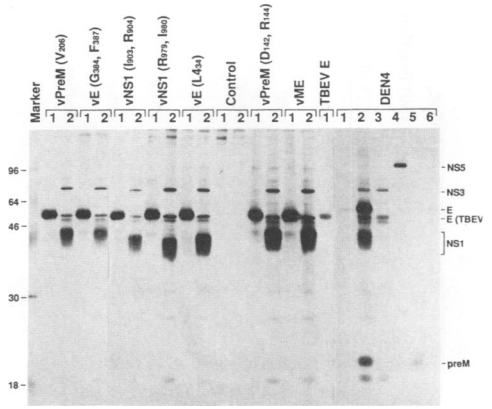

FIG. 1. Analysis of viral proteins produced in DEN4- or chimeric ME virus-infected cells. [^{35}S]methionine-labeled lysates of virus-infected or uninfected (control) simian cells (as indicated on top) were immunoprecipitated with TBEV HMAF (lanes 1), DEN4 HMAF (lanes 2), or rabbit serum specific to NS3, NS5, pre-M, or E (lanes 3 to 6, respectively) of DEN4 and analyzed by SDS-PAGE and autoradiography. ^{14}C-labeled E protein from purified virions of TBEV was immunoprecipitated with TBEV HMAF under same conditions; this protein was provided by E. K. Pressman (Novosibirsk Institute of Bioorganic Chemistry). Molecular sizes of protein markers are given in kilodaltons at left. Locations of DEN4 proteins and TBEV E protein are indicated at right.

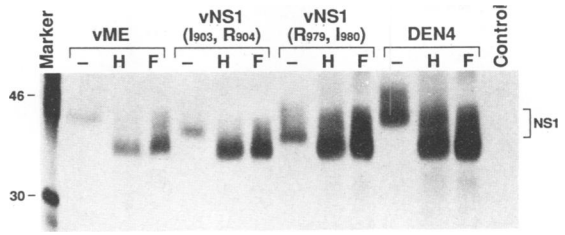

FIG. 2. Endoglycosidase treatments on intracellular NS1 protein of DEN4, vME, and vNS1 mutants. Radioactive-labeled lysates of vME, DEN4, vNS1(I_{903}, R_{904}), vNS1(R_{979}, I_{980}), or uninfected (control) LLC-MK_2 cells were immunoprecipitated with DEN4 NS1-specific monoclonal antibody 8E2-2 (lanes –). Immunoprecipitated NS1 was digested with endo H (20 U/ml, lanes H) or endo F (1 U/ml, lanes F). The samples were analyzed by SDS-PAGE. Numbers on left show size in kilodaltons.

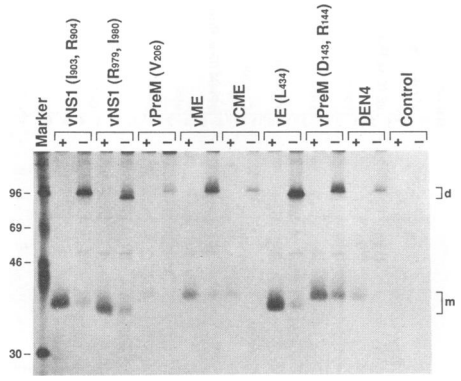

FIG. 3. Analysis of intracellular NS1 protein dimers in virus-infected LLC-MK$_2$ cells. ^{35}S-labeled lysates of virus-infected or uninfected (control) LLC-MK$_2$ cells were immunoprecipitated with monoclonal antibody 8E2-2 specific for DEN4 NS1 protein. Samples were analyzed by SDS-PAGE with (+) or without (−) prior boiling. Locations of monomeric (m) or dimeric (d) forms of NS1 are indicated at right. Numbers on left show size in kilodaltons.

dimers, we analyzed the NS1 immunoprecipitates of both NS1 mutants by PAGE with or without prior boiling (Fig. 3). A protein band migrating at about 90 to 96 kDa predicted to be dimeric NS1 was detected in unboiled samples. However, following boiling, the complex dissociated to yield monomers of about 41 kDa, suggesting that only one carbohydrate chain is required for dimer formation.

The E proteins of vE(L$_{434}$) and parental vME virus did not appear to differ in gel migration. Also, gel mobility of the E protein produced by mutant vE(L$_{434}$) or parental vME did not differ following treatment with endo F or endo H (Fig. 4); both E products were sensitive to endoglycosidase digestion. This is surprising because, according to the published data, the TBEV E protein contains an asparagine-linked carbohydrate at the first glycosylation site (11, 25, 27), and the mutation in vE(L$_{434}$) should abolish this site. Thus, the glycosylation mutation of vE(L$_{434}$) did not completely ablate glycosylation of the E protein. Analysis of sequence of TBEV E has identified several other potential N-linked glycosylation sites, and these are located at Asn-641-Pro-Thr, Asn-753-Pro-Thr, and Asn-383-His-Cys (21). Possibly, one of these otherwise nonused glycosylation sites became available for glycosylation when the preferred glycosylation site was abolished. Also unexpected was the abnormal gel migration of the NS1 protein produced by vE(L$_{434}$) (Fig. 1 and 4). However, after endoglycosidase treatment, the NS1 protein of vE(L$_{434}$) and parental vME exhibited the same gel mobility (Fig. 4). Sequence analysis of the NS1 gene in the regions between nts 2745 and 2881 and between nts 2993 and 3165 of this chimera did not reveal any nucleotide changes. It is possible that glycosylation of the NS1 protein of vE(L$_{434}$) was also altered by a conformational change of the NS1 protein resulting from ablation of the E glycosylation site. If this is the case, there may be a close interaction between E and NS1 glycoproteins.

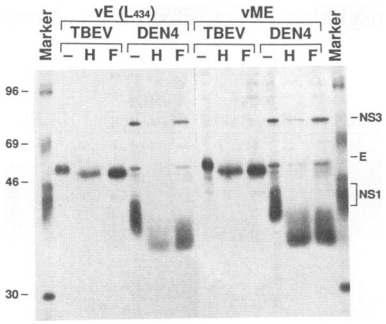

FIG. 4. Effect of endoglycosidase treatment on immunoprecipitated TBEV E protein and DEN4 nonstructural proteins produced by chimeric vME- or vE(L$_{434}$)-infected cells. ^{35}S-labeled lysates of vME- or vE(L$_{434}$)-infected LLC-MK$_2$ cells were immunoprecipitated with TBEV or DEN4 HMAF as indicated. Immunoprecipitates were treated by endo H (lanes H) or endo F (lanes F) or not subjected to digestion (lanes −) and then analyzed by SDS-PAGE. Numbers on left show size in kilodaltons.

TABLE 2. Characteristics of TBE(ME)/DEN4 chimeric virus mutants[a]

Virus	Plaque size (mm) on:		Mouse neurovirulence LD_{50} (PFU)	*P* value of difference from vME
	LLC-MK$_2$ cells	C6/36 cells		
DEN4	1.8	11.6	>10,000	
vME	5.5	6.9	20	
vCME	1.3	7.0	240	<0.001
vPreM(V_{206})	1.8	5.5	3,800	<0.001
vPreM(D_{143}, R_{144})	4.5	4.0	1	<0.001
vE(L_{434})	0.4	2.5	2,040	<0.001
vE(G_{384}, F_{387})	4.0	6.2	2	<0.001
vNS1(I_{903}, R_{904})	0.5	2.7	380	<0.001
vNS1(R_{979}, I_{980})	0.3	2.2	3	0.02

[a] Plaque morphology assay of mutant viruses was performed as described previously (2, 20). Monolayers of LLC-MK$_2$ or C6/36 cells inoculated with virus were overlaid with agar and stained with neutral red 6 days later. Mouse LD_{50} and *P* value were estimated after i.c. inoculation of each virus in a dose from 0.1 to 10,000 PFU as described in Materials and Methods.

Plaque morphology and growth properties in cell culture. The mutant chimeras were compared to each other and to the parental chimeric viruses as regards plaque morphology and pattern of replication in cell culture (Table 2 and Fig. 5). Replication of vME and vCME on C6/36 cells did not differ, nor was plaque size different. However, vCME produced smaller plaques than vME on LLC-MK$_2$ cells, and similarly, the maximum yield of vCME at 6 days after infection was lower than that of vME in these cells. This indicates that vCME is more restricted in growth in mammalian cells than vME. With the exception of vE(G_{384}, F_{387}), mutant chimeric viruses produced plaques that were considerably smaller than those produced by vME on LLC-MK$_2$ or C6/36 cells (Table 2). Plaque size reduction of these mutants was more profound on LLC-MK$_2$ cells than on C6/36 cells. Also, mutations that were introduced to disrupt glycosylation of pre-M, E, or NS1 or cleavage of pre-M reduced growth in cell culture (Fig. 5). Similar to parental vME, each of the mutants replicated faster and attained a higher titer in simian cells than in mosquito cells. Compared with parental vME, the growth rate of vPreM(V_{206}) and vE(L$_{434}$) mutants was found to be reduced in both cell lines. Six days after infection, mutants vPreM(V_{206}) and vE(L$_{434}$) grew to a level 1/100th or 1/1,000th that of vME in LLC-MK$_2$ cells. Similarly these mutants attained a maximal titer 1/10th or 1/100th that of vME in C6/36 cells. These observations, together with differences in plaque size, support the view that cleavage site mutation in vPreM(V_{206}) and glycosylation site mutation of vE(L$_{434}$) decreased the efficiency of viral repli-

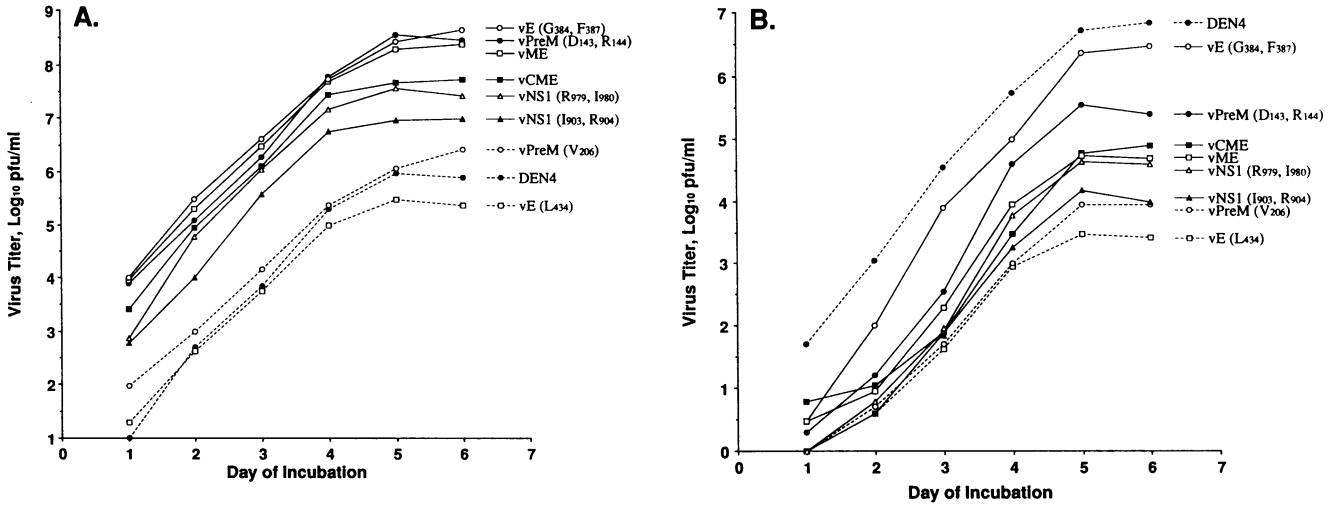

FIG. 5. Growth of chimeric TBE(ME)/DEN4 virus mutants in simian or mosquito cells. LLC-MK_2 (A) or C6/36 (B) cells were harvested at indicated times (days after infection by indicated virus at 0.01 PFU per cell), and the virus titer was determined by a plaque assay on the respective cells (2).

cation. Compared with vME, the NS1 glycosylation site mutation in vNS1(I_{903}, R_{904}) also caused a greater reduction of viral growth than did the NS1 glycosylation site mutation in vNS1(R_{979}, I_{980}). Also, growth of vME and vPreM(D_{143}, R_{144}) in LLC-MK_2 cells was similar. In contrast, the mutation introduced into vE(G_{384}, F_{387}) increased viral yield of the mutant virus in both cell lines. This mutant reached a titer about 60-fold higher on C6/36 cells than that attained by parental vME under the same conditions.

Mouse neurovirulence. In a previous experiment, vME did not cause encephalitis in mice when inoculated by a peripheral route at a dose of 10^3 PFU (20). To test the possibility that a higher dose of vME may breach the blood-brain barrier and cause fatal encephalitis, we inoculated 6-week-old mice with a larger dose of vME, i.e., 10^4, 10^5, 10^6, or 10^7 PFU by the i.p. or i.m. route. While all eight mice succumbed to encephalitis between days 10 and 16 after i.c. inoculation of 10^4 PFU, mice inoculated i.p. or i.m. with up to 1,000-fold more virus remained healthy during the 28-day observation period. This indicates that unlike TBEV, which is highly pathogenic for mice by peripheral inoculation (i.p. 50% lethal dose [LD_{50}] of TBEV for adult BALB/c mice is 14.2 PFU) (20), chimeric vME virus, even at a dose of 10^7 PFU, failed to produce central nervous system disease. This leads us to conclude that the neuroinvasiveness of vME for mice is greatly reduced. However, the chimera retains the neurovirulence phenotype of its TBEV parent when inoculated i.c.

To evaluate the effect of the various mutations on neurovirulence, we inoculated 6-week-old BALB/c mice in groups of eight i.c. with each mutant virus, in a calculated dose ranging from 0.1 to 10,000 PFU per animal and observed them for 31 days for signs of encephalitis and death. This experiment was performed twice for vE(L_{434}) and vNS1(I_{903}, R_{904}), three times for vPreM(V_{206}), and four times for vME and DEN4 in order to achieve a more accurate estimate of mouse neurovirulence. Minimal variation of mortality (0 to 15%) was observed in replicate experiments. The LD_{50} for each virus was calculated (7), and the value was used as the basis for comparison of mouse neurovirulence. Mice inoculated with DEN4 did not develop encephalitis. The LD_{50} of vPreM(D_{143}, R_{144}), vNS1(R_{979}, I_{980}), or vE(G_{384}, F_{387}) was significantly reduced compared with the LD_{50} of 20 PFU for the parental vME. The observed increase of neurovirulence was 20-, 6.6-, and 10-fold, respectively. The mean survival time for mice inoculated with these three mutants was shorter than that of mice given the same dose of vME, confirming that these mutants are highly virulent. In contrast, the calculated mouse neurovirulence of vNS1(I_{903}, R_{904}) and vCME was more than 10 times less than that of vME. Of greater interest was the finding that mutants vPreM(V_{206}) and vE(L_{434}) were more than 100 times less neurovirulent than vME. Those mutants showing reduction of neurovirulence also exhibited reduced growth in simian and mosquito cell culture (Table 2 and Fig. 5). Two of the mutants, i.e., vPreM(D_{143}, R_{144}) or vE(G_{384}, F_{387}), that exhibited increased neurovirulence in mice replicated to higher titer in mosquito cells than did parental vME. However, increased growth was not observed in simian cells.

Antibody response and protective efficacy. The mouse encephalitis model was used to determine the protective efficacy of the vME mutants. Five mice were inoculated with 10^4 PFU i.p. or i.m. or 10 PFU i.c. of each mutant. All mice remained healthy without signs of encephalitis 21 days following i.p. or i.m. inoculation. In contrast, a few deaths were observed in mice inoculated i.c. with one of the following mutants: vPreM(V_{206}), vNS1(R_{979}, I_{980}), vPreM (D_{143}, R_{144}), and vE(G_{384}, F_{387}). On day 22 postinfection, sera from all i.p. or i.m. inoculated mice and from the survivors of i.c. inoculation were analyzed by radioimmunoprecipitation assay in the presence of an excess of labeled antigens and electrophoresis on SDS-PAGE (Fig. 6). The intensity of label present in the TBEV E or DEN4 NS1 protein bands provided an estimate of the level of E and NS1 specific antibodies induced by each mutant. Interestingly, antibodies to E or NS1 were low or not detectable in sera of mice inoculated with vE(L_{434}) or vNS1(R_{979}, I_{980}), indicating that chimeric mutants containing amino acid substitutions at the glycosylation site of E or NS1 were less immunogenic than the unmodified vME virus. Like parental vME, the vPreM(V_{206}) and vNS1(I_{903}, R_{904}) induced readily detectable amounts of antibodies to E or NS1.

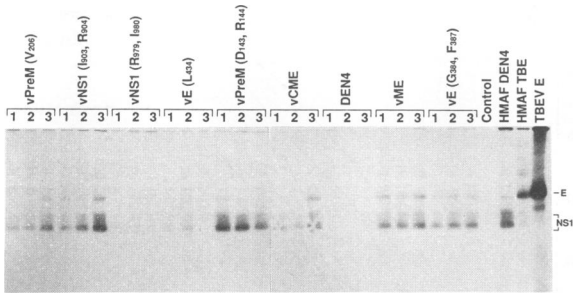

FIG. 6. Immunoresponse following i.p., i.m., or i.c. inoculation of mice with mutant viruses. Mice were inoculated i.p. (lanes 1) or i.m. (lanes 2) with 10^4 PFU of virus or i.c. (lanes 3) with 10 PFU and were bled 22 days after infection. Serum from all inoculated mice in each group were combined, and 10 μl of this serum pool was used for immunoprecipitation of ^{35}S-labeled proteins from a lysate of vME-infected LLC-MK_2 cells. As a positive control for immunoprecipitation, TBEV or DEN4 HMAF was used. The samples were analyzed by SDS-PAGE. Lane labeled TBEV E indicates location of ^{14}C-labeled envelope protein from virions of TBEV which was immunoprecipitated under the same conditions as TBEV HMAF.

Twenty-five days after i.p. or i.m. inoculation with a mutant virus, vME, vCME, or DEN4, surviving mice were challenged i.c. with 10^4 PFU (approximately 500 LD_{50}) of vME. All mice that were previously immunized i.p. or i.m. with vME, vCME, or a mutant vME were completely protected against i.c. challenge of vME. In contrast, mice immunized i.p. or i.m. with DEN4 were only partially protected: two of five died in the i.p.-inoculated group and three of five in the i.m.-inoculated group between days 15 and 20. All nonimmunized control mice died of encephalitis between days 12 and 18 following challenge. These results indicate that antibodies against DEN4 nonstructural proteins, including the known protective antigen NS1 (6, 8), were not able to completely protect mice. In contrast, the TBEV pre-M and E proteins induced resistance to lethal i.c. challenge with vME. The level of antibodies specific to the TBEV E or DEN4 NS1 protein did not correlate with resistance to i.c. challenge. For example, the immune response of mice which were inoculated peripherally with vE(L_{434}) or vNS1(R_{979}, I_{980}) was poor. However, these mice remained completely healthy without any symptoms of disease during 4 weeks after i.c. challenge with neurovirulent vME virus.

DISCUSSION

Live attenuated strains of yellow fever virus, Japanese encephalitis virus, and DEN2 virus have been successfully developed by serial passage of the wild-type virulent virus in animals or cultured cells that are not natural hosts to the virus. However, development of other flavivirus vaccines by this procedure has met with only limited success. Presently, a licenced live TBEV or dengue virus vaccine is not available. In an effort to understand the mechanism of flavivirus attenuation, complete nucleotide and encoded amino acid sequences of the attenuated strain of yellow fever virus, Japanese encephalitis virus, and dengue virus were compared with the sequences of the virulent parental virus (1, 3, 12, 19). In each instance, numerous amino acid changes were identified that were widely dispersed among the structural and nonstructural proteins. At this time, the genetic determinants of attenuation of the licensed or experimental live flavivirus vaccines have not been elucidated.

To expedite the development of a live attenuated TBEV vaccine, we constructed a novel chimeric TBE(ME)/DEN4 virus and used the general strategy of engineering defined mutations in various regions of the chimeric viral genome at the cDNA level. Our intent was to recover virus mutants that could be evaluated for attenuation by the mouse experimental encephalitis model. In this study, we chose to mutate the coding sequence of TBEV pre-M or E protein because observations made during a previous study indicated that these two TBEV structural proteins possess most or all of the determinants of mouse neurovirulence as assayed by direct i.c. inoculation. Also, these proteins, as well as the NS1 protein of DEN4, which was also selected for mutational analysis, undergo posttranslational modification or secondary cleavage which can be specifically interdicted by mutation. In addition, the TBEV pre-M and E structural proteins and the DEN4 NS1 nonstructural protein are N-glycosylated, which permitted us to evaluate the effect of ablation of glycosylation on neurovirulence.

A total of six viable mutants of chimeric vME were recovered. These included a mutant in which (i) the TBEV pre-M cleavage site was ablated [vPreM(V_{206})], (ii) the TBEV pre-M glycosylation site was ablated [vPreM(D_{143}, R_{144})], (iii) the first dengue virus NS1 glycosylation site was ablated [vNS1(I_{903}, R_{904})], (iv) the second dengue virus NS1 glycosylation site was ablated [vNS1(R_{979}, I_{980})], (v) the TBEV E glycosylation site was ablated [vE(L_{434})], or (vi) two amino acid substitutions in TBEV changed position 384 to the conserved mosquito-borne flavivirus sequence and changed position 387 to the attenuated TBEV sequence [vE(G_{384}, F_{387})].

Only three of the mutants, i.e., vPreM(V_{206}), vE(L_{434}), and vNS1(I_{903}, R_{904}), exhibited restriction of replication in both simian LLC-MK_2 and mosquito C6/36 cells. Significantly, each of the three mutants exhibited a considerable decrease in neurovirulence as assayed by direct i.c. inoculation. Three other mutants exhibited an increase in neurovirulence. None of these mutants was restricted in replication in both cell lines. However, one of these mutants [vNS1(R_{979}, I_{980})] was restricted in simian cells, but the degree of restriction was less than that observed for the three attenuated mutants.

Reduction in plaque size was not tightly associated with reduction in neurovirulence. For example, although two of the three attenuated mutants produced small plaques in both cell lines, the third mutant produced small plaques on simian cells but not mosquito cells. In addition, one of the mutants that appeared to be more neurovirulent [i.e., vNS1(R_{979}, I_{980})] produced small plaques on both simian and mosquito cells. Thus, the only conserved feature of those examined for the three attenuated mutants was restriction of virus yield in both simian and mosquito cells.

Our present study identified three mutations that are each associated independently with a significant reduction of mouse neurovirulence of vME. These mutations ablate (i) the TBEV pre-M cleavage site, (ii) the TBEV E glycosylation site, or (iii) the first DEN4 NS1 glycosylation site. In contrast, ablation of the second DEN4 NS1 glycosylation site or the TBEV pre-M glycosylation site or amino acid substitution at two positions in the TBEV E protein increased neurovirulence.

vME was previously shown to lack neuroinvasiveness. By this we mean the inability to spread from a peripheral site of inoculation to the central nervous system and thereby cause

fatal encephalitis. In a previous study, vME failed to produce fatal encephalitis in mice when inoculated i.p. or intradermally at a dose of 10^3 PFU. Lack of neuroinvasiveness was confirmed in the present study and could not be breached by increasing the dose of virus to 10^7 PFU. Our success in achieving significant reduction of neurovirulence means that the three attenuated mutants generated in this study are now doubly attenuated since they are unable to spread from a peripheral site to the brain and, if this did occur, the likelihood of disease would be considerably reduced. Significantly, parenteral inoculation of each of these doubly attenuated mutants induced complete resistance to subsequent i.c. challenge with neurovirulent vME.

Clearly, the next step in our strategy to develop a safe and effective live attenuated TBEV vaccine will be to continue mutational analysis of the TBE/DEN4 chimeric virus with the intent of constructing mutant chimeras containing a combination of two or all three of the mutations that were shown to independently reduce neurovirulence. This approach may yield a mutant chimeric virus with the desired level of attenuation and protective efficacy. Also, chimeric viruses bearing multiple mutations offer the potential advantage of greater genetic stability.

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