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Construction and characterization of chimeric tick-borne encephalitis/dengue type 4 viruses

(dengue virus/tick-borne encephalitis virus/viral chimera/neurovirulence/protective immunity)

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ABSTRACT **Dengue type 4 virus (DEN4) cDNA was used as a vector to express genes of the distantly related tick-borne encephalitis virus (TBEV). Full-length chimeric TBEV/DEN4 cDNAs were constructed by substituting TBEV genes coding for proteins such as capsid (C); pre-membrane, which is the precursor of membrane (M); envelope (E); or nonstructural protein NS1 for the corresponding DEN4 sequences. RNA transcripts prepared from cDNAs were used to transfect permissive simian cells. Two viable chimeric viruses that contained TBEV CME or ME genes were recovered. Compared with DEN4, chimeric TBE(ME)/DEN4 virus [designated vTBE(ME)/DEN4] produced larger plaques and grew to higher titer in simian cells. In contrast, vTBE(ME)/DEN4 produced smaller plaques on mosquito cells and grew to lower titer than DEN4. Analysis of viral RNA and proteins produced in vTBE(ME)/DEN4- and DEN4-infected mosquito or simian cells revealed that the chimera was restricted in its ability to enter and replicate in mosquito cells. In contrast, vTBE(ME)/DEN4 entered simian cells efficiently and its RNA was replicated more rapidly in these cells than was parental DEN4 RNA. Following intracerebral inoculation, vTBE(ME)/DEN4 caused fatal encephalitis in both suckling and adult mice, while nearly all mice inoculated by the same route with DEN4 did not develop disease. Unlike wild-type TBEV, vTBE(ME)/DEN4 did not cause encephalitis when adult mice were inoculated by a peripheral route. Adult mice previously inoculated with the chimera by a peripheral route were completely resistant to subsequent intraperitoneal challenge with 10^3 times the median lethal dose of TBEV, whereas mice previously inoculated with DEN4 were not protected. These findings indicate that (*i*) the TBEV M and E genes of the chimeric virus are major protective antigens and induce resistance to lethal TBEV challenge and (*ii*) other regions of the TBEV genome are essential for the ability of this virus to spread from a peripheral site to the brain. Success in constructing a viable TBEV/DEN4 chimera that retains the protective antigens of TBEV but lacks its peripheral invasiveness provides a strategy for the development of live attenuated TBEV vaccines.**

Many members of the *Flaviviridae* family cause significant public health problems in different regions of the world (1). Tick-borne encephalitis virus (TBEV) causes a serious encephalitic illness with a mortality ranging from 1% to 30%. Currently, a vaccine produced by formalin inactivation of TBEV is available, but this vaccine has several limitations, including the need for repeated vaccination, and low protective efficacy. With the exception of the yellow fever virus 17D vaccine that is used extensively throughout the world, attempts to produce an effective live attenuated vaccine against other flaviviruses have not yielded licensed products. Elucidation of the organization of the flavivirus genome and identification of its protective antigens have led to the development of several flavivirus vaccine strategies which include immunization with (*i*) a live recombinant vaccinia virus expressing one or more flavivirus protective antigens (2–7) or (*ii*) a lysate of moth cells infected with a recombinant baculovirus expressing similar antigens (8). These approaches have not generated an acceptable candidate vaccine. Another approach to flavivirus vaccine development was made possible by construction of cloned full-length cDNA that could be transcribed to yield infectious RNA (9, 10). This makes it possible to construct defined virus mutants.

Following success in the construction of full-length infectious dengue type 1/type 4 and dengue type 2/type 4 intertypic chimeric viruses (11), we initiated the construction of chimeric viruses containing sequences from dengue type 4 virus (DEN4) and the distantly related TBEV, which belongs to another serotype complex of the *Flaviviridae* (12). DEN4 and TBEV have the same genome organization and share the same strategy of gene expression, but comparison of sequences between the two viruses indicates that the homology is relatively low (13). The immediate objective of our study was to determine whether any TBEV/DEN4 gene constellation could produce a viable chimeric virus and to characterize those viable chimeric viruses that were recovered. Two chimeric viruses that contained the capsid/membrane/envelope (CME) or ME structural protein genes of TBEV were viable and exhibited the antigenicity of TBEV.

MATERIALS AND METHODS

Chimeric TBEV/DEN4 cDNA. Previously, subgenomic cDNA fragments of TBEV (strain Sofjin) were cloned and the nucleotide sequence was determined (13). Plasmids pGEM2-CME, containing nucleotides (nt) 76–1977, and pGEM2-ENS1, containing nt 966–3672 of the TBEV sequence, were constructed by E. Yu. Dobricova (Novosibirsk Institute of Bioorganic Chemistry) from plasmids p10, p4, p18, p2, and p11 (13) by joining at shared restriction enzyme sites. Plasmids DEN4 p5′-2 and p5′-2(Δ*Pst* I, *Xho* I) (11) and a derivative, p5′-2(Δ*Pst* I, *Xho* I, Δ*Hin*dIII), were used to substitute one or more TBEV genes for the corresponding DEN4 gene(s). Sequences at the junctions between TBEV and DEN4 genes in each chimeric plasmid were confirmed.

Chimeric Viruses. Transcription reaction and the DNase treatment of the transcription mixture were performed essentially as described (10). The RNA transcripts were then used to transfect simian LLC-MK_2 cells in the presence of (*i*) Lipofectin (Bethesda Research Laboratories), as described (10), or (*ii*) *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) (Boehringer Mann-

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Abbreviations: TBEV, tick-borne encephalitis virus; DEN4, dengue type 4 virus; IFA, immunofluorescence assay; HMAF, hyperimmune mouse ascitic fluid; nt, nucleotide(s); pfu, plaque-forming unit(s); i.c., intracerebral(ly); i.d., intradermal(ly); i.p., intraperitoneal(ly).

heim). In the latter case, the transfection mixture contained RNA transcripts (2 μg) in 40 μl of 0.02 mM Hepes buffer, pH 7.05, and 12 μl of DOTAP in 30 μl Hepes buffer. After incubation at room temperature for 10 min, 2 ml of medium 199 containing 10% fetal bovine serum was added; 0.5 ml of the mixture was distributed to subconfluent cells in 4 wells of a 24-well plate. Ten days later, cells were trypsinized and transferred to a 6-well plate and chamber slides for an additional 2 days of incubation in growth medium. Cells on the slides were tested for the presence of DEN4 or TBEV antigens by immunofluorescence assay (IFA) using a 1:100 dilution of DEN4 hyperimmune mouse ascitic fluid (HMAF), TBEV HMAF, or TBEV-specific rabbit serum. Fluorescein-conjugated anti-mouse or anti-rabbit serum (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used at the same dilution. When IFA indicated that 50–80% of cells were infected, cells in the 6-well plate were trypsinized, mixed with a 2-fold excess of uninfected cells, and grown in a 75-cm^2 tissue culture flask for 6 days. The infected cells were then harvested together with the medium, mixed with an equal volume of fetal bovine serum, and used as the source of progeny chimeric virus. Parental DEN4 and its chimeric viruses were characterized by plaque assay on simian LLC-MK_2 and mosquito C6/36 cells (11, 17).

To verify the genomic structure of chimeric TBE(ME)/DEN4 virus [vTBE(ME)/DEN4], RNA isolated from infected LLC-MK_2 cells was reverse-transcribed by using an oligonucleotide that is complementary to the DEN4 sequence at nt 5090–5110 (14). The single-stranded cDNA was employed as the template for PCR using the primer pair corresponding to the DEN4 sequence at nt 18–44 (15) and the TBEV sequence at nt 2129–2150 (13), or to the DEN4 sequence at nt 3460–3480 and the TBEV sequence at nt 967–984. The PCR DNA products were each tested for the cleavage-site sequence by restriction enzyme digestion. Sequence of the junction between the C gene of DEN4 (300–398 nt) and the pre-M gene of TBEV (418–460 nt) was also confirmed by sequencing the cloned DNA fragment.

To analyze proteins produced by vTBE(ME)/DEN4 or DEN4 [v2A(*Xho* I)] recovered from full-length cDNA (11), confluent LLC-MK_2 cells in a 6-well plate were infected with the respective virus at a multiplicity of infection of 0.01. Six days after infection, cells were labeled with [^{35}S]methionine (60 μCi per well, specific activity 600 Ci/mmol; 1 Ci = 37 GBq) in methionine-free MEM (Eagle's minimal essential medium) for 4 hr as described (10). Lysates were immunoprecipitated with HMAF or specific serum as indicated in the figure legend. Immunoprecipitates were analyzed by SDS/PAGE (16).

Analysis of Viral RNA and Protein Synthesis. LLC-MK_2 or C6/36 cells in 25-cm^2 tissue culture flasks were infected with vTBE(ME)/DEN4 or DEN4 at a multiplicity of infection of 1. After adsorption at 37°C for 1 hr, virus inoculum was removed and fresh medium was added to the cells. For analysis of protein synthesis, infected cells at various times (0, 4, 8, 24, and 48 hr) following virus adsorption were incubated with methionine-free MEM for 20 min and labeled with [^{35}S]methionine in the same medium for 2 hr. The lysate of labeled cells was precipitated with DEN4 HMAF and analyzed by PAGE and fluorography.

For analysis of viral RNA synthesis, ≈10^6 infected LLC-MK_2 or C6/36 cells were collected at various times (0, 4, 8, 24, and 48 hr) following virus adsorption, rinsed with 0.5 ml of phosphate-buffered saline (PBS, pH 7.4), and then lysed in 0.3 M NaOAc, pH 5.2/5 mM EDTA/1% SDS. Total RNA was isolated from the cell lysate and the medium by phenol extraction. RNA samples were denatured by incubation in 6× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7)/7.5% (wt/vol) formaldehyde at 60°C for 15 min and bound to a BA85 nitrocellulose filter (Schleicher & Schuell). Filters were baked at 80°C for 1 hr and prehybridized in 6× SSC/3× Denhardt's solution/25 mM Na_2HPO_4, pH 6.5/0.1% SDS containing salmon sperm DNA (25 μg/ml) at 65°C for 1 hr. Hybridization was continued overnight at 65°C in the same solution containing a nick-translated, ^{32}P-labeled pTBE(ME)/DEN4 DNA probe (50 ng/ml; specific activity, 3.4 × 10^8 cpm/μg). After hybridization, the filters were washed five times in 0.1× SSC/0.1% SDS at 65°C, dried, and exposed to an x-ray film at −70°C. The radioactivity of [^{32}P]DNA hybridized to RNA was also measured in a liquid scintillation counter.

Mouse Neurovirulence of Chimeric vTBE(ME)/DEN4 and Parental DEN4. vTBE(ME)/DEN4 and DEN4 were analyzed for neurovirulence by inoculating mice intracerebrally (i.c.), intradermally (i.d.), or intraperitoneally (i.p.). Three-day-old suckling BALB/c mice were injected i.c. with 10^2 plaque-forming units (pfu) of virus in 0.02 ml of MEM/0.25% human serum albumin. Six-week-old BALB/c female mice were (*i*) inoculated i.c. with 10^3 pfu of virus, diluted as above, in a volume of 0.03 ml, or (*ii*) inoculated i.d. or i.p. with 10^3 pfu of virus, diluted in 0.10 ml. Mice were observed for 21 days for symptoms of encephalitis or death, and surviving adult mice were bled 20 days after infection to evaluate antibody response. Surviving mice were challenged i.p. at 21 days with 10^3 LD_{50} (median lethal dose) of TBEV (strain Sofjin).

RESULTS

Recovery of Chimeric Viruses. With pGEM2-CME or pGEM2-ENS1 as the template, a series of TBEV cDNA fragments that define one or more specific genes flanked by restriction enzyme cleavage sites were prepared by PCR with oligonucleotide primers. Table 1 shows seven such TBEV cDNA fragments and their terminal sequences for joining to the appropriate sites similarly introduced into the DEN4 moiety during chimeric cDNA construction. RNA transcripts derived from each of the seven full-length chimeric TBEV/DEN4 cDNA templates were tested for infectivity by transfection of LLC-MK_2 cells. In six separate transfection experiments conducted using Lipofectin, only the TBE(ME)/DEN4 RNA yielded progeny virus identifiable by IFA. Cells transfected with RNA transcripts of pTBE(ME)/DEN4 were stained with TBEV-specific rabbit serum or HMAF or DEN4-specific HMAF. DEN4-infected cells were not stained by TBEV-specific serum. This indicated that chimeric vTBE(ME)/DEN4 expressed both TBEV- and DEN4-specific antigens.

Transfection of cells with the chimeric cDNA-derived RNA transcripts was also conducted using *N*-[1-(2,3-dioleoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP). Sixteen days after transfection with TBE(CME)/DEN4 RNA, ≈1% of cells stained positively for TBEV- and DEN4-specific antigens in two separate experiments. The percentage of positive cells increased to 30% by day 20, and 80% by day 26, at which time the titer of virus in transfected cells was 6 × 10^5 pfu/ml. After transfection with TBE(ME)/DEN4 RNA, the percentage of IFA-positive cells was 60–80% at day 10 and increased to 100% by day 16, at which time the titer of virus present in transfected cells was 4 × 10^6 pfu/ml. The titer of virus produced by cells transfected with DEN4 RNA transcripts was 5.1 × 10^5 pfu/ml, as observed earlier (15).

Additional evidence that progeny virus was derived from infectious TBE(ME)/DEN4 RNA transcripts was provided by analysis of a cloned DNA fragment of the progeny virus genome, which confirmed the junction sequence between DEN4 C and TBEV pre-M genes (Table 1).

Viral Proteins of Chimeric vTBE(ME)/DNA. Proteins produced in chimeric or parental DEN4 virus-infected cells were precipitated with specific antibodies and analyzed by PAGE (Fig. 1). Both chimeric virus and parental virus produced protein bands identified as DEN4 NS3 and NS5 (lanes 3 and 4). DEN4 pre-M or E antiserum precipitated the respective protein band only from DEN4-infected cells (lanes 5 and 6),

Table 1. Intergenic junctions in chimeric TBEV/DEN4 constructs

Construct	Amino acid/nucleotide sequences	TBEV cDNA*
pTBE(CME)/DEN4	M A ... L N S R N CAG ATC CTG GGG ATG GCC ... CTG AAC TCG AGG AAC BglII/BamHI XhoI	121-2376
pTBE(ME)/DEN4	R K R S A V ... L N S R N AGA AAA AGG TCT GCA GTA ... CTG AAC TCG AGG AAC PstI XhoI	415-2376
pTBE(MENS1)/DEN4	R K R S A V ... M V H A G Q AGA AAA AGG TCT GCA GTA ... ATG GTG CAT GCC GGA CAG PstI SphI	415-3504
pTBE(E)/DEN4	S Y G S R C ... L N S R N TCC TAC GGA TCT CGG TGC ... CTG AAC TCG AGG AAC BamHI/BglII XhoI	967-2376
pTBE(ENS1)/DEN4	S Y G S R C ... M V H A G Q TCC TAC GGA TCT CGG TGC ... ATG GTG CAT GCC GGA CAG BamHI/BglII SphI	967-3504
pTBE(NS1)/DEN4	G T N S R N ... M V H A G Q GGC ACG AAC TCG AGG AAT ... ATG GTG CAT GCC GGA CAG XhoI SphI	2380-3504
pTBE(C)/DEN4	M A ... K R R S A V CAG ATC CTG GGG ATG GCC ... AAA AGA AGG TCT GCA GTA BglII/BamHI PstI	121-417

DEN4 and TBEV genome have the same gene order: 5′-C–pre-M–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5-3′ (C, capsid protein; pre-M, precursor of membrane protein; E, envelope protein; NS, nonstructural protein). Restriction enzyme-cleaved TBEV cDNA fragments were inserted into DEN4 cDNA at appropriate sites as indicated by the underlined sequence. The amino acids and the encoding nucleotide sequences of TBEV are in bold letters.
*Nucleotide numbering system is from ref. 13.

but not from chimeric virus-infected cells. DEN4-specific HMAF precipitated protein bands only from vTBE(ME)/DEN4-infected cell lysate that comigrated with DEN4 NS1 and NS3 (lane 2). On the other hand, TBEV HMAF immunoprecipitated from the lysate of chimeric virus-infected cells a protein identified as TBEV E (about 55 kDa) migrating faster than DEN4 E (lanes 1 and 6). The TBEV-specific HMAF preparation failed to precipitate TBEV pre-M and C in previous tests, providing an explanation for why TBEV-specific pre-M was not detected (lane 1). TBEV E was completely sensitive to endoglycosidase F or H digestion; a reduction of 2–3 kDa in molecular mass was observed (data not shown). The profile of protein bands produced in LLC-MK_2 cells by vTBE(CME)/DEN4 was identical to that produced by vTBE(ME)/DEN4. Thus, the chimeric viruses produced the expected proteins.

Plaque Morphology. DEN4 on mosquito C6/36 cells produced plaques with an average size of 12.1 mm, whereas vTBE(ME)/DEN4 produced plaques averaging 6.5 mm (Fig. 2*A*). In contrast, on simian LLC-MK_2 cells vTBE(ME)/DEN4 produced plaques that were 5-fold larger than those produced by DEN4. This suggested that the chimeric virus replicated more efficiently in LLC-MK_2 cells than did DEN4. This was confirmed by analysis of the growth rate and the viral yield of vTBE(ME)/DEN4 in infected LLC-MK_2 cells. The chimeric virus reached a titer of 10^8 pfu/ml, ≈1000-fold higher than that attained by parental DEN4, v2A(*Xho* I), under the same conditions (Fig. 2*B*). In contrast, chimeric vTBE(ME)/DEN4 grew slowly on mosquito C6/36 cells and reached a titer 100 times lower than that produced by parental DEN4. Plaque size of chimeric vTBE(CME)/DEN4 did not differ appreciably from that of DEN4 on LLC-MK_2 cells (data not shown).

Viral RNA and Protein Synthesis. Analysis of viral RNA and proteins provided an explanation for the small-plaque phenotype and the reduced growth rate of vTBE(ME)/DEN4 in infected C6/36 cells. DEN4 viral proteins, including E, NS1, and NS3, accumulated to a high level in DEN4-infected LLC-MK_2 or C6/36 cells by 48 hr after infection (Fig. 3*A*). However, in vTBE(ME)/DEN4-infected LLC-MK_2 and C6/36 cells the kinetics of viral protein synthesis differed. Viral proteins were detected as early as 8 hr postinfection in LLC-MK_2 cells, whereas viral proteins were not detected in C6/36 cells until 48 hr after infection. We also analyzed viral RNA synthesis in parental DEN4- or chimeric virus-infected cells. Following 1 hr of virus adsorption, RNA was isolated at various times from the infected cells and analyzed by hybridization with a radioactive DNA probe (Fig. 3*B*). Approximately 70% of the vTBE(ME)/DEN4 virions remained in the medium following inoculation onto C6/36 cells, whereas only a small fraction of the inoculated virus was found in medium of LLC-MK_2 cell cultures. This suggests

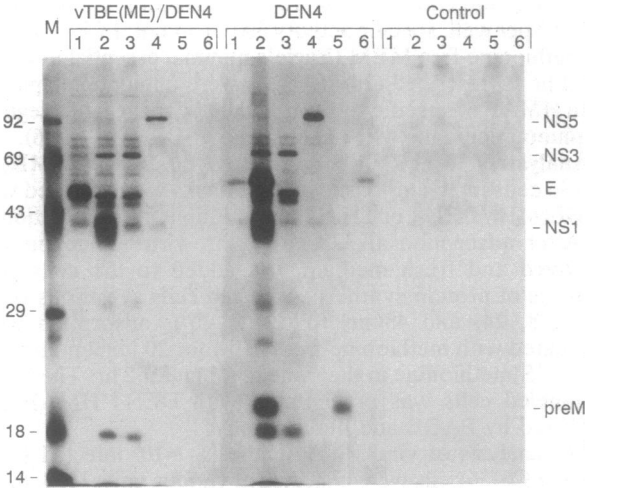

FIG. 1. Analysis of viral proteins produced by parental DEN4 and chimeric TBE(ME)/DEN4 viruses. [^{35}S]Methionine-labeled lysates of vTBE(ME)/DEN4- or DEN4-infected or uninfected (control) simian cells were immunoprecipitated using TBEV HMAF (lanes 1), DEN4 HMAF (lanes 2), or rabbit serum specific to NS3, NS5, or pre-M (lanes 3–5, respectively), or E (lanes 6) of DEN4 and analyzed by SDS/12% PAGE followed by autoradiography. Molecular sizes of protein markers are (lane M) given in kilodaltons at left. Locations of DEN4 proteins are indicated at right.

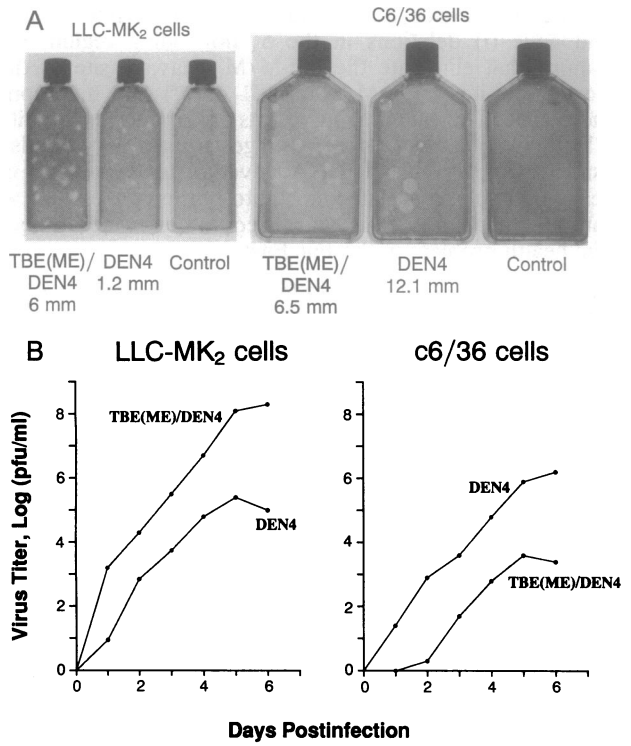

FIG. 2. Plaque morphology and growth analysis of vTBE(ME)/DEN4 and DEN4 on LLC-MK_2 and C6/36 cells. (*A*) Monolayers of LLC-MK_2 or C6/36 cells inoculated with DEN4 or vTBE(ME)/DEN4 were overlaid with agar and stained with neutral red 6 days later. (*B*) Cells were harvested at indicated times (days after infection at 0.01 pfu/cell), and the virus titer was determined by a plaque assay on the respective cells (11, 17).

that entry of chimeric vTBE(ME)/DEN4 into LLC-MK_2 cells was more efficient than was entry into C6/36 cells. Following entry into LLC-MK_2, replication of chimeric viral RNA was more rapid than that of DEN4 RNA. On the other hand, RNA synthesis of chimeric virus was slower than that of DEN4 in infected C6/36 cells. Thus, vTBE(ME)/DEN4 exhibited reduced efficiency of entry into mosquito cells that was associated with a reduced production of viral RNA and proteins. These findings are consistent with the low efficiency of transfection of C6/36 cells by TBEV RNA (18).

Neurovirulence, Immunogenicity, and Protective Efficacy of Chimeric vTBE(ME)/DEN4 in Mice. vTBE(ME)/DEN4 retained the neurovirulence of its TBEV parent when inoculated directly into the brain (i.c.) of suckling or adult mice (Table 2). TBEV is highly neurovirulent and 0.1 pfu readily caused fatal encephalitis in 50% of suckling mice inoculated i.c. TBEV is also highly virulent for adult mice inoculated by a peripheral route; the i.p. LD_{50} was 14.2 pfu. In contrast, vTBE(ME)/DEN4 did not cause encephalitis when inoculated by a peripheral route, that is, i.d. or i.p. (Table 2).

The immunogenicity of vTBE(ME)/DEN4 was analyzed by immunoprecipitation of ^{35}S-labeled antigens by serum of surviving mice. Analysis of the immunoprecipitates by PAGE revealed that antibodies specific to DEN4 NS1 (a protein encoded by the chimeric virus) were readily detected, but antibodies to TBEV E or M were of low titer or not detectable (data not shown). Twenty-one days after inoculation with vTBE(ME)/DEN4 or DEN4 virus, surviving mice were challenged i.p. with 10^3 LD_{50} of the highly neurovirulent Sofjin strain of TBEV. The mice that survived i.p. or i.d. inoculation with chimeric vTBE(ME)/DEN4 were protected against subsequent challenge, whereas all three groups of mice previously immunized with DEN4 died between day 11 and day 20 (Table 2). Nonimmunized control mice died of

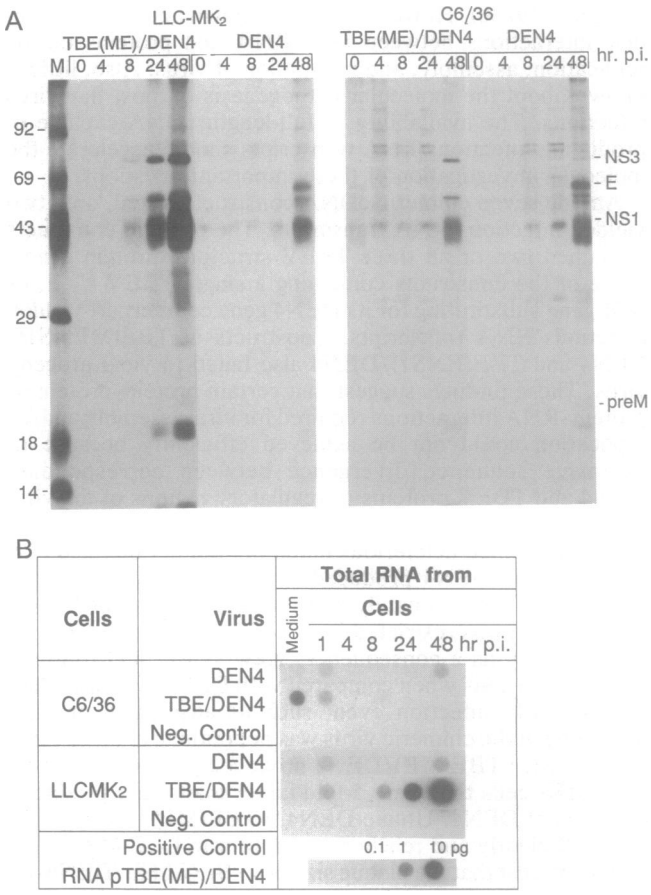

FIG. 3. RNA and protein synthesis in vTBE(ME)/DEN4- and DEN4-infected LLC-MK_2 or C6/36 cells. (*A*) Viral protein synthesis at various times postinfection (p.i.) was analyzed as described in *Materials and Methods*. (*B*) RNA was analyzed by dot blot hybridization with a [^{32}P]cDNA probe.

encephalitis between day 10 and day 16 after TBEV infection (data not shown). These findings indicate that vTBE(ME)/DEN4 contains major antigenic determinants of resistance against TBEV encephalitis in mice.

DISCUSSION

Substantial progress has been made in defining flavivirus genome organization, viral polyprotein processing, and function of viral proteins associated with the flavivirus replication

Table 2. Neurovirulence and protective immunity of vTBE(ME)/DEN4 in mice

Virus	Age of mice	Route of inoculation	Mortality after inoculation*	Mortality after challenge*
vTBE(ME)/ DEN4	3 days	i.c.	8/8	—
	6 weeks	i.c.	5/5	—
		i.d.	0/5	0/5
		i.p.	0/5	0/5
DEN4	3 days	i.c.	1/8	Not tested
	6 weeks	i.c.	0/5	5/5
		i.d.	0/5	5/5
		i.p.	0/5	5/5

Suckling BALB/c mice were inoculated i.c. with 10^2 pfu and 6-week-old mice inoculated i.c., i.p., or i.d. with 10^3 pfu of the indicated virus. Mice were observed for signs of encephalitis or death for 21 days. Surviving mice were challenged i.p. with 10^3 LD_{50} of TBEV (strain Sofjin) and observed for 4 weeks.
*No. of mice that died/no. of mice tested.

complex (19–30). However, little is known about the molecular interactions between viral RNA and proteins during replication, assembly, and maturation of virus. Also, little is known about the molecular pathogenesis of most flavivirus infections. The availability of full-length cDNA capable of producing infectious RNA transcripts should accelerate the molecular investigation of these important questions.

Among seven chimeric cDNA constructs tested, only two yielded infectious RNA transcripts. The positive constructs contained two or all three TBEV structural protein genes. None of the constructs containing a single TBEV C, E, or NS1 gene substituting for its DEN4 gene counterpart yielded infectious RNA transcripts. Constructs of TBE(MENS1)/DEN4 and TBE(ENS1)/DEN4 also failed to yield progeny virus. These findings suggest that certain protein–protein or protein–RNA interactions required for viral assembly and/or replication could not be achieved efficiently because of extensive sequence divergence between corresponding DEN4 and TBEV proteins or regulatory regions of the viral genomes. However, the possibility that these cDNA constructs contained deleterious mutations cannot be ruled out.

vTBE(ME)/DEN4 produced small plaques on mosquito C6/36 cells, approximately half the size of those of DEN4. This chimera was restricted in its ability to enter C6/36 cells and, possibly as a consequence, grew slower and to lower titer in these cells when compared with DEN4. We speculate that an early infection event such as adsorption or viral uncoating of the chimeric virus was not optimal in these cells. In contrast, vTBE(ME)/DEN4 produced plaques on simian LLC-MK_2 cells that were 5-fold larger than those produced by parental DEN4. Unlike DEN4, vTBE(ME)/DEN4 replicated efficiently and reached a high titer in LLC-MK_2 cells. It is of interest that the plaque size of vTBE(CME)/DEN4 did not differ appreciably from that of DEN4 on LLC-MK_2 cells. This may reflect an incompatibility of TBEV C protein with DEN4 RNA. It is also possible that this difference is the result of substitution of the six nucleotides upstream of the AUG codon in the DEN4 5′ noncoding region, which may influence the efficiency of translation. TBE(ME)/DEN4 virus uniformly caused encephalitis in both suckling and adult mice following i.c. inoculation, whereas mice inoculated with DEN4 developed this disease with low frequency. Thus, the chimeric virus retained the mouse neurovirulence of TBEV from which its pre-M and E genes were derived. This indicates that most, if not all, of the genetic determinants of TBEV mouse neurovirulence map within these two structural protein genes. However, unlike parental TBEV, vTBE(ME)/DEN4 was not pathogenic when adult mice were inoculated peripherally, indicating a loss of neuroinvasiveness. These findings suggest that a region of the TBEV genome other than the pre-M and E genes is required for this virus to invade the central nervous system and produce encephalitis. Mice inoculated peripherally with vTBE(ME)/DEN4 were protected against subsequent i.p. challenge with a lethal dose of TBEV, whereas mice similarly inoculated with DEN4 were not. This observation indicates that TBEV pre-M, M, and/or E proteins are major determinants of protective immunity.

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, an additional modification of the chimera must be achieved—namely, ablation of neurovirulence as measured by direct inoculation of virus into the brain. Since the TBEV/DEN4 chimera retains the neurovirulence of its TBEV parent, it will be necessary to abolish this property by engineering strategic mutations in the DEN4 or TBEV portion of the chimeric genome and evaluating their effect on mouse neurovirulence. Such mutant TBE(ME)/DEN4 viruses would have (*i*) deletions in the 5′ noncoding region, (*ii*) mutations that eliminate the pre-M/M cleavage site or the glycosylation sites of pre-M, E, or NS1, or (*iii*) point mutations in the E gene. The encouraging results observed thus far with the TBEV(ME)/DEN4 chimera suggest that it may be also possible to employ DEN4 cDNA as vector for expression of genes of more closely related mosquito-borne viruses such as Japanese encephalitis virus, which continues to be a major public health problem in the Far East.

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