Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 1746–1751, February 1998
Medical Sciences

Attenuation of the Langat tick-borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4

(viral chimeras/neurovirulence/neuroinvasiveness/protective immunity)

ALEXANDER G. PLETNEV* AND RUHE MEN

Molecular Viral Biology Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Communicated by Robert M. Chanock, National Institutes of Health, Bethesda, MD, December 9, 1997 (received for review November 11, 1997)

ABSTRACT **Langat virus (LGT) strain TP21 is the most attenuated of the tick-borne flaviviruses for humans. Even though LGT has low-level neurovirulence for humans, it, and its more attenuated egg-passage derivative, strain E5, exhibit significant neurovirulence and neuroinvasiveness in normal mice, albeit less than that associated with tick-borne encephalitis virus (TBEV), the most virulent of the tick-borne flaviviruses. We sought to reduce or ablate these viral phenotypes of TP21 and E5 by using a strategy that had been used successfully in the past to reduce neurovirulence and abolish neuroinvasiveness of TBEV, namely substitution of structural protein genes of the tick-borne flavivirus for the corresponding genes of dengue type 4 virus (DEN4). In pursuit of these objectives different combinations of LGT genes were substituted into the DEN4 genome but only chimeras containing LGT structural proteins premembrane (preM) and envelope glycoprotein (E) were viable. The infectious LGT(preM-E)/DEN4 chimeras were restricted in replication in simian cell cultures but grew to moderately high titer in mosquito cell culture. Also, the chimeras were at least 5,000 times less neurovirulent than their parental LGT virus in suckling mice. Significantly, the chimeras lacked detectable evidence of neuroinvasiveness after i.p. inoculation of Swiss mice or the more permissive SCID mice with 10^5 or 10^7 plaque-forming units (PFU), respectively. Nonetheless, i.p. inoculation of Swiss mice with 10 or 10^3 PFU of either chimeric virus induced LGT neutralizing antibodies and resistance to fatal encephalitis caused by i.p. challenge with LGT TP21. The implications of these observations for development of a live attenuated TBEV vaccine are discussed.**

Tick-borne flaviviruses are endemic throughout most of the Northern Hemisphere, causing disease of varying severity that can have a mortality as high as 20–30% (1). Similar to all flaviviruses, viruses of the tick-borne group have a positive sense nonsegmented RNA genome that encodes a single long polyprotein that is processed to yield capsid (C), premembrane (preM), envelope glycoprotein (E) structural proteins followed by nonstructural protein NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 in that order (2, 3). These tick-borne viruses share envelope glycoprotein epitopes that often induce cross-resistance among viruses of the group. These properties of antigenic crossreactivity and virulence polymorphism suggested that successful immunization might be achieved by using a live, naturally attenuated tick-borne flavivirus. The impetus for this approach was the recovery of a virus from ticks in Malaysia, namely Langat virus (LGT), that did not appear to be associated with human disease under natural conditions (4, 5).

Approximately 30 years ago Yelantsev virus (6), which subsequently was shown to be identical to wild-type LGT, strain TP21 (7), was evaluated in 649,479 individuals as a candidate live attenuated vaccine for prevention of tick-borne encephalitis. Studies were discontinued when it was discovered that vaccination was associated with a low frequency of encephalitis, approximately one case per 18,570 immunizations. At about that time, a mutant of LGT TP21 that exhibited less virulence for mice and monkeys was selected by 42 passages in embryonated chicken eggs (8–10).

Even though wild-type LGT (TP21) had very low virulence for humans, this virus and its more attenuated derivative (E5) exhibited significant neurovirulence in mice, albeit somewhat less than that of tick-borne encephalitis virus (TBEV). In addition, LGT TP21 exhibited neuroinvasiveness (''peripheral virulence''), but this was significantly reduced compared with TBEV. Finally, LGT E5 showed very little evidence of neuroinvasiveness in mice (9).

Before evaluating the more attenuated E5 mutant of LGT as a possible candidate for use in prophylaxis of severe disease caused by certain members of tick-borne flavivirus group, we considered it desirable to remove the last vestiges of virulence of LGT E5 for experimental animals. The approach we used to achieve this objective was that recently developed for the successful attenuation of TBEV (11). This strategy involved the creation of a viable chimera in which genes for structural proteins preM and E of TBEV were substituted for the corresponding genes of dengue type 4 virus (DEN4). The resulting TBEV/DEN4 chimera exhibited a reduction in neurovirulence for mice, as tested by intracerebral inoculation. However, more impressive was the effect on neuroinvasiveness that reflects the capacity of virus to replicate at a peripheral site and then spread to the central nervous system where encephalitis is produced. Chimerization of TBEV with DEN4 completely ablated detectable neuroinvasiveness so that peripheral inoculation of 10^7 plaque-forming units (PFU) of the chimera did not produce encephalitis, a 714,000-fold reduction of neuroinvasiveness (12).

In the present study, the same strategy was applied to LGT E5, as well as its TP21 parent for the purpose of comparison, and was found to be effective. In addition, a considerable increase in ability to detect neuroinvasiveness was achieved by using SCID mice rather than immunocompetent mice. When this more sensitive system for measuring ''peripheral virulence'' was used, we still were unable to detect any evidence of this form of virulence in either LGT/DEN4 chimera.

MATERIALS AND METHODS

Viruses. The LGT wild-type strain TP21 strain used in this study was kindly provided by R. Shope (University of Texas Medical Branch, Galveston) from the Rockefeller Foundation

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/98/951746-6$0.00/0
PNAS is available online at http://www.pnas.org.

Abbreviations: LGT, Langat virus; DEN4, dengue type 4 virus; PFU, plaque-forming unit; IC, intracerebral(ly); TBEV, tick-borne encephalitis virus; preM, premembrane; MOI, multiplicity of infection.

*To whom reprint requests should be addressed at: Building 7, Room 236, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 7 Center Dr MSC 0740, Bethesda, MD 20892. e-mail: apletnev@atlas.niaid.nih.gov.

Collection and originally was isolated from ticks in Malaya in 1956 (4). It had undergone nine passages in mouse brain and two in *Vero* cell cultures when received by us. The LGT strain E5 (provided by J. Huggins, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD) was derived from the TP21 strain by 42 passages in 7-day-old chicken embryos and had an additional passage in simian *Vero* cells (8, 9). Both LGT viruses were plaque-purified three times on Vero cells under soft agar before preparation of virus stocks.

Genome Sequences of Langat TP21 and E5 Viruses. Purification of LGT strains and isolation of their RNA were performed as described previously for TBEV (3). First-strand cDNA was synthesized by using an oligonucleotide primer complementary to the conserved 22 3′-terminal nucleotides of TBEV strains (13–15). PCR then was used to amplify overlapping cDNA fragments of the LGT TP21 or E5 genome. The sequences of the PCR primers were derived from the published coding region of LGT TP21 strain (7, 16). The PCR fragment corresponding to the 5′-noncoding region was generated by using a primer that contained the first 21 conserved 5′-terminal nucleotides of the TBEV genome (13, 14, 17). Finally, the sequence of the 5′- and 3′-end of LGT TP21 or E5 genome was confirmed when 5′ and 3′ termini of viral RNA were ligated, and cDNA fragments corresponding to the 5′- and 3′-terminal genomic regions were generated by the PCR using a protocol published by Mandl *et al.* (18). Each PCR product was cloned in *Escherichia coli* BD1528 using a *Hin*dIII and a *Pvu*I cleavage site of the p5′-2 (*Not*I, *Xho*I, Δ*Hin*dIII) vector (19). The complete nucleotide sequence of the TP21 and E5 genomes was determined by sequencing both strands of overlapping cDNA clones that were derived from two or more independently prepared cDNAs.

Recovery of Chimeric LGT/DEN4 Viruses. Plasmid DEN4 p2A(*Xho*I) (20) containing the DEN4 cDNA and pTBEV-(preME)/DEN4 (11) containing the TBEV(preME)/DEN4 chimera sequence were used to substitute two or more LGT genes for the corresponding DEN4 genes (Table 1). Oligonucleotide-directed mutagenesis was performed to introduce a *Cla*I site instead of the unique Asp-718 site at the 3′ end of dengue sequence in p2A(*Xho*I). To facilitate construction of chimeric LGT TP21(CpreME)/DEN4 or E5(CpreME)/DEN4 cDNA, the cDNA segment encoding the DEN4 C, preM, and E genes, extending from *Bgl*II (nucleotide 88) to the *Xho*I site (nucleotide 2,342) was replaced with the corresponding sequence of TP21 or E5 virus. To construct chimeric LGT(preME)/DEN4 cDNA containing the preM and E genes of the LGT wild-type TP21 strain or its more attenuated E5 derivative, four different junctions between the DEN4 C gene and LGT preM gene were created in chimeric DNA plasmids (Table 1). Sequences at the junctions between LGT and DEN4 genes in each chimeric plasmid were verified. LGT(nonstructural protein NS1 and NS2A; NS1, NS2A, NS2B and dNS3; and NS2B and NS3)/DEN4 chimeras were constructed in a similar manner.

RNA transcripts of the full-length LGT/DEN4 constructs listed in Table 1 were used to transfect simian Vero or $LLCMK_2$ cells or mosquito C6/36 cells in presence of DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate) (11, 12). On day 12 and again on days 16, 20, 24, 28, 32, 36, and 44, cells were split and passaged. Cells were examined on each of these occasions by immunofluorescence assay for the presence of DEN4 and LGT antigens using a DEN4- or LGT-specific hyperimmune mouse ascitic fluid. Only two infectious chimeric viruses were isolated. These viruses were recovered from transfected mosquito cells, namely, TP21/DEN4, which contains LGT TP21 preM and E genes, and E5/DEN4, which contains LGT E5 preM and E genes. The recovered chimeras were amplified once in mosquito C6/36 cells, and viral RNA was isolated and reverse-transcribed with oligo 2634 (12). Sequence of the LGT/DEN4 junction sites and sequence of LGT preM and E genes was confirmed by direct analysis of the cloned DNA inserts.

The procedures used for plaque assay, growth curve analysis, and labeling of viral proteins with L-[^{35}S] methionine were described earlier (11, 12). Radioimmunoprecipitation of viral

Table 1. Intergenic junctions in chimeric LGT TP21/DEN4 and LGT E5/DEN4 constructs

Construct	Amino acid/nucleotide sequences	LGT cDNA nt	Viability
pLGT(CpreME)/DEN4	**M A G K G L N S R** GAG CAG ATC TCT GGA AAA **ATG GCC GGG AAG**... ...**GGC TTG AAC TCG AGG** *Bgl*II *Xho*I	129-2379	No
pLGT(preME)/DEN4	L Q **R R G S R R T G L N S R** 1. CTG CAG **CGC CGA GGA AGT AGA AGG ACG**... ...**GGC TTG AAC TCG AGG** *Pst*I *Xho*I	403-2379	No
	N G R K R S I I D G L N S R 2. AAC GGG AGA AAA AGA TCG ATC **ATT GAC**... ...**GGC TTG AAC TCG AGG** *Cla*I *Xho*I	426-2379	No
	N G R K R S A V D G L N S R 3. AAC GGG AGA AAA AGG TCT GCA G**TT GAC**... ...**GGC TTG AAC TCG AGG** *Pst*I *Xho*I	428-2379	Yes
	A F S L V A R E R G L N S R 4. GCG TTT TCC TTG GTT GCA **AGA GAG AGA**... ...**GGC TTG AAC TCG AGG** *Bst*BI *Xho*I	490-2379	No
pLGT(NS1,2A)/DEN4	T N S **R N P T R G R R S** W P ACG AAC TCG **AGG AAC CCA ACC**... ...**AGG GGG AGA CGA TCG** TGG CCT *Xho*I *Pvu*I	2382-4205	No
pLGT(NS1,2A,2B,d3)/DEN4	N S **R N P T G T G W** I R K AAC TCG AGG **AAC CCA ACC**... ...**GGC ACG GGC TGG A**TT CGA AAG *Xho*I *Bst*BI	2382-5161	No
pLGT(NS2B,3)/DEN4	A S R **R S F N E S G R** R S I GCC TCA AGA **CGA TCG TTT AAT GAG**... ...**TCT GGA AGA** AGA TCT ATA *Pvu*I *Bgl*II	4201-6456	No

The terminal sequences of the corresponding cDNA fragments of LGT TP21 and LGT E5 are identical. Restriction enzyme-cleaved LGT TP21 or E5 cDNA fragments were inserted into DEN4 cDNA at appropriate sites as indicated by the underlined sequence. The amino acid and the encoding nucleotide sequences of LGT TP21 are in bold letters. (Nucleotide numbers for E5 are one less because of 5′ noncoding region deletion.) Infectivity of RNA transcripts from DNA constructs was tested by transfecting simian and mosquito cells and evaluating cell cultures for evidence of infection by immunofluorescence assay.

proteins was performed with LGT TP21- or DEN4-specific hyperimmune mouse ascitic fluid and mAbs specific for TP21 preM or E protein (21).

Evaluation of Parental and Chimeric Viruses in Mice. The neurovirulence of LGT/DEN4 chimeras and their parental viruses was assessed in 3-day-old outbred Swiss mice. Groups of 10–14 mice were inoculated intracerebrally (IC) with decimal dilutions of virus ranging from 10^{-2} to 10^{7} PFU and observed for 28 days for fatal or nonfatal encephalitis.

Neuroinvasiveness of parental and chimeric viruses was evaluated in 3-week-old female Swiss mice that were inoculated by the i.p. route in groups of 10 with: (*i*) 10, 10^2, 10^3, 10^4, 10^5, 10^6, or 10^7 PFU of LGT TP21 virus or E5 virus or (*ii*) 10^5 PFU of TP21/DEN4, E5/DEN4, or DEN4 virus and observed for 21 days. Survivors were bled to evaluate antibody response, challenged i.p. the next day with 100 or 1,000 i.p. LD_{50} of TP21 virus, and observed for an additional 4 weeks. A more sensitive assay for neuroinvasiveness was developed by using immunodeficient (SCID) mice. In this assay female 3-week-old C.B.-17 ICR/*scid*/*scid* mice (22) in groups of five were inoculated i.p. with: (*i*) 10^7 PFU of DEN4, TP21/DEN4, or E5/DEN4 virus or (*ii*) decimal dilutions of LGT TP21 or E5 ranging from 10^{-4} to 10^7 PFU. These mice then were observed for mortality for 6 weeks.

To determine whether the protective efficacy and immunogenicity of chimeric TP21/DEN4 and E5/DEN4 viruses observed in the initial protection study reflected an effect of preformed nonreplicating viral antigens, five female 3-week-old Swiss mice were inoculated i.p. with: (*i*) 10, 10^3, or 10^5 PFU of TP21/DEN4 or E5/DEN4 virus or (*ii*) with the same amount of virus that had been completely inactivated by UV light. Under the conditions used for UV inactivation, 60 min was sufficient to completely inactivate infectivity as determined by plaque assay on mosquito C6/36 cells. Mice were observed 21 days, and survivors were bled 22 days after inoculation to evaluate antibody response measured by ELISA or a plaque reduction-neutralization test. Surviving mice were challenged i.p. at 24 days with 1,000 i.p. LD_{50} (5×10^6 PFU) of LGT TP21 and observed for 4 weeks.

RESULTS

Comparison of Nucleotide and Amino Acid Sequences of Wild-Type LGT Virus (Strain TP21) and its Derivative (Strain E5). The genome of wild-type LGT, strain TP21, as well as its more attenuated derivative, strain E5, were cloned and sequenced to identify the boundaries of LGT genes used to construct chimeric viruses. Sequence analysis also allowed us to identify mutations that were potentially responsible for differences in biological properties of the parental virus and its mutant. The TP21 or E5 genome was 10,940 or 10,941 nucleotides in length, respectively, and contained a single ORF coding for a 3,414-aa polyprotein. The LGT E5 5′ noncoding region (NCR) was 130 nt in length and differed from the 5′ NCR of TP21 virus by deletion of G at nucleotide position 61 and by presence of a C residue instead of a G at position 35. The 3′ NCR of LGT E5 strain was 566 nt in length and differed from its TP21 parent by the insertion of a dinucleotide (AC) between positions 10,515 and 10,516 and by deletion of C and U at positions 10,599 and 10,633, respectively.

There were 24 nucleotide differences between TP21 and E5, of which 11 produced an amino acid substitution in the polyprotein (Table 2). Amino acid changes were located in the C and E structural proteins and the NS1, NS2A, and NS3 nonstructural proteins. Sequence analysis of separate TP21 cDNA clones, which coded for the E protein gene, revealed that a substitution $C_{1436} > U$ was present in three of four clones. Interestingly, mutation Asn-668 > Asp in E protein of strain E5 corresponds to a substitution observed previously in the E protein of a partially attenuated mutant of TBEV (23, 24).

Construction of LGT/DEN4 Chimeric Genomes and Test for Viability. Viable LGT/DEN4 chimeras might allow us to alter neurovirulence, tissue tropism, and peripheral invasiveness of LGT TP21 and E5 as well as define compatibility of certain mixed LGT/DEN4 gene constellations. Full-length DEN4 cDNA was used to engineer chimeric constructs containing the LGT C-preM-E, preM-E, NS1-NS2A, NS1-NS2A-NS2B-dNS3, or NS2B-NS3 genes with the remaining sequences derived from DEN4 (Table 1). Full-length RNA transcripts made from these chimeric cDNA templates then were tested for infectivity by transfecting simian $LLCMK_2$, simian Vero or mosquito C6/36 cells. Only two viable chimeric viruses were recovered. These viruses contained preM and E genes of TP21 or E5 and were recovered only after transfection of mosquito C6/36 cells. Repeated attempts to recover these two chimeric viruses in simian cells failed.

Table 2. Differences in nucleotide and amino acid sequences between Langat virus strains TP21 and E5

Strain	E5		TP21	
Gene	NT	AA	NT	AA
C	C_{371}	Pro_{80}	A	Thr
	A_{461}	Ile_{110}	C	Leu
preM	U_{514}		G	
E	A_{1327}		C	
	A_{1342}		G	
	C_{1436}	Thr_{435}	C/U	Thr/Ile
	U_{1567}		A	
	A_{1823}	Ser_{564}	G	Gly
	C_{1968}	Ser_{612}	U	Phe
	G_{2135}	Asp_{668}	A	Asn
NS1	A_{3008}	Met_{960}	G	Val
	U_{3403}		C	
NS2A	G_{3635}	Ala_{1168}	C	Pro
	C_{3637}		G	
	A_{3964}		G	
NS3	G_{4662}	Ser_{1510}	A	Asn
	A_{5339}	Tyr_{1736}	U	Phe
	U_{5374}		C	
	C_{5546}	Leu_{1805}	U	Phe

NT or AA is indicated nucleotide or amino acid residue at indicated position of LGT E5 genome of polyprotein. Polyprotein sequence of strain TP21 determined in this study differed from that of the TP21 polyprotein published earlier (7, 16). Instead of Glu-152, Ser-564, Ser-612, Met-960, Ala-1168, Ile-2149, Val-2357, Ser-2775, Ala-2921, Cys-3157, and Val-3158, we found a Asp, Gly, Phe, Val, Pro, Met, Ile, Cys, Gly, Trp, or Leu residue, respectively.

Sequence analysis of the TP21/DEN4 and E5/DEN4 chimeras confirmed the junction sequences between DEN4 C and LGT preM genes or LGT E and DEN4 NS1 genes. Importantly, the chimeras differed in their LGT-derived sequences at only four amino acid positions in the E protein (Table 2). In addition, immunoprecipitation of viral proteins from infected mosquito cell culture indicated that both of these chimeras produced the expected proteins (data not shown).

Growth of Chimeras in Cell Culture. The TP21/DEN4 and E5/DEN4 chimeras were compared with each other and to their parental viruses with respect to pattern of replication and maximum yield in simian and mosquito cells (Fig. 1). When inoculated at a multiplicity of infection (MOI) of 0.01, the chimeras grew to a moderate titer in mosquito cells, namely $10^{4.8}$ or $10^{6.0}$ PFU/ml. In contrast, the growth of their parental LGT TP21 or E5 virus in mosquito cells was totally restricted. This restriction was observed when a MOI ranging from 0.01 to 1,000 was used. In addition, immunofluorecence assay failed to detect evidence of viral replication in any of these instances. The titer attained by the chimeras in mosquito cells on day 5 was 10- to 100-fold reduced compared with DEN4. Unlike DEN4, the two chimeras induced a chronic infection of mosquito cells.

A different hierarchy of viral replication was observed when simian cells were used as host cells. Chimerization of LGT TP21

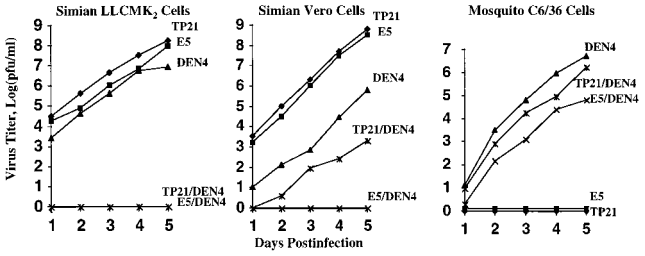

FIG. 1. Growth of parental and chimeric viruses in simian and mosquito cells. Chimeric virus inocula were grown in mosquito cells, parental LGT TP21 and E5 virus inocula were grown in simian Vero cells, whereas DEN4 parental virus inoculum for mosquito cells was grown in mosquito cells and DEN4 inoculum for simian cells was grown in simian Vero cells. Simian LLCMK$_2$ or Vero cells were infected with: (*i*) DEN4, TP21, or E5 virus at MOI of 0.01 or (*ii*) chimeric TP21/DEN4 or E5/DEN4 virus at MOI of 0.5. Mosquito C6/36 cells were infected with: (*i*) DEN4, TP21/DEN4, or E5/DEN4 virus at MOI of 0.01 or (*ii*) with TP21 or E5 virus at MOI of 1,000. Cells were harvested at indicated time (day after infection), and the virus titer was determined by a plaque assay on the same cells used for study of virus replication. Plaques were enumerated after 7 or 8 days of infection. In other experiments complete restriction of LGT TP21 and E5 in mosquito cells also was observed when MOI ranging from 0.01 to 1,000 were used.

or E5 with DEN4 significantly reduced the efficiency of viral replication in simian cells compared with parental TP21 or E5 virus as well as DEN4. As a consequence, the LGT/DEN4 chimeric viruses grown in mosquito cells were not able to produce plaques in simian cells at 28°C, 32°C, or 37°C; thus the observed restriction was not a manifestation of a temperature-sensitive phenotype. In addition, these chimeras did not replicate efficiently in simian cells as indicated by plaque assay of growth yield in mosquito cells (data not shown). In contrast, parental LGT TP21 or E5 was able to produce plaques with high efficiency and grew to high titer in simian cells, i.e., $\approx 10^8$ PFU/ml.

Simian cells were not completely refractory to the chimeric viruses because virus propagated in permissive mosquito cell culture did initiate slow and partially restricted viral replication in simian LLCMK$_2$ or Vero cells inoculated at a MOI of 0.5. In addition, spread of virus in these cell cultures differed from that of parental TP21 or E5 virus that were cytopathic, progressed rapidly, and attained a high titer by day 5 when inoculated at a MOI of 0.01. In contrast, infection of simian cells with either chimera at a MOI of 0.5 did not produce cytopathic effects and progressed very slowly as monitored by immunofluorescence assay. Twenty-eight to 48 days of incubation were required for 80–100% of simian cells to become infected. At this time a chronic infection without cytopathic effect became established and such chronically infected cells could be maintained during incubation and subculture for 10 months without apparent visible effect. Virus yield from these simian cell cultures at the time of maximum infection was measured by plaque assay on mosquito cells and found to be reduced by 90% from the level attained when either chimeric virus was grown in mosquito cells. The yield of either chimeric virus from infected simian LLCMK$_2$ cells did not produce plaques on these cells. This finding was also the case for the E5/DEN4 chimera grown and titered in simian Vero cells. In contrast, a reduced number of very small faint plaques was produced by the TP21/DEN4 chimera in these cells, a finding consistent with the limited replication of the chimera in simian cells. Long-term growth of chimeric viruses in simian cells led to selection of heterogeneous plaque-size mutants that were detectable on mosquito C6/36 cells.

These observations indicate that chimerization of LGT TP21 or E5 with DEN4 significantly reduced the replicative capacity of these chimeras in simian cells compared with either parental virus. This finding is in contrast to our previous observation that chimera TBEV(ME)/DEN4 replicated 1,000 times more efficiently in simian cells than did DEN4 (11, 12).

Mouse Neurovirulence of LGT/DEN4 Chimeras. Wild-type LGT strain TP21 was highly neurovirulent with an IC LD_{50} of 0.4 PFU in suckling mice (Table 3). Neurovirulence of the more attenuated LGT strain E5 by this route was 50 times less (20 PFU). DEN4 was even less neurovirulent with an IC LD_{50} of 8,000 PFU. Both chimeric TP21/DEN4 and E5/DEN4 exhibited a significant reduction in neurovirulence compared with its LGT parent. Thus, the IC LD_{50} of TP21/DEN4 or E5/DEN4 was 5,500- to 6,250-fold greater than that of its LGT parent. In addition, chimera E5/DEN4 was 44 times less neurovirulent than chimera TP21/DEN4. It appeared that the TP21/DEN4 and E5/DEN4 chimeric viruses retained the low mouse neurovirulence of their DEN4 parent rather than the higher mouse neurovirulence of their LGT parent.

Peripheral Virulence ("Neuroinvasiveness") of LGT/DEN4 Chimeras. LGT TP21 was moderately virulent for 3-week-old mice when inoculated by a peripheral route; its i.p. LD_{50} was 5×10^3 PFU (Table 3). In contrast, chimeric TP21/DEN4 exhibited lower peripheral neuroinvasiveness for adult mice with an i.p. LD_{50} of $>10^5$ PFU. The attenuated E5 strain exhibited lower peripheral virulence than its TP21 parent. Only 10–20% of adult mice inoculated i.p. with 10^7 PFU of E5 showed symptoms of encephalitis. This made it difficult to detect an effect of chimerization on neuroinvasiveness. On the other hand, the data regarding TP21/DEN4 indicated that chimerization of TP21 with DEN4 effected a significant reduction in neuroinvasiveness of this tick-borne flavivirus for mice.

Subsequent studies performed in SCID mice indicated that these immunodeficient animals represented a considerably more sensitive test system for detection of neuroinvasiveness (Table 3). For example, the i.p. LD_{50} of either parental LGT virus in SCID mice was 0.06–0.004 PFU, which was at least 10^6 times less than observed in immunocompetent mice. In contrast, both chimeric viruses lacked detectable neuroinvasiveness when the highly sensitive SCID mice were inoculated i.p. with 10^7 PFU. This represented an overall decrease in this property of greater than 1.7×10^8- to 2.5×10^9-fold compared with LGT parental virus. The current observations are consistent with earlier findings that chimerization of TBEV with DEN4 significantly reduced its neuroinvasiveness for mice (12).

Table 3. Neurovirulence and neuroinvasiveness of parental and chimeric viruses in immunocompetent or SCID mice

	Neurovirulence for immunocompetent mice IC LD_{50} (PFU)		Neuroinvasiveness: i.p. LD_{50} (PFU)			
			Immunocompetent mice		SCID mice	
Virus	Unmodified virus	(preME)/DEN4 chimera	Unmodified virus	(preME)/DEN4 chimera	Unmodified virus	(preME)/DEN4 chimera
TBEV*	0.1	20	14	>10,000,000	Not tested	Not tested
LGT TP21	0.4	2,500	5,000	>100,000	0.004	>10,000,000†
LGT E5	20	110,000	>10,000,000	>100,000	0.060	>10,000,000†
DEN4	8,000	—	>10,000,000	—	>10,000,000	—

*Data from ref. 12 provided for purpose of comparison.
†Virus was concentrated by centrifugation at 40,000 × *g* onto a 60% sucrose cushion.

Table 4. Antibody response and protective efficacy of chimeric LGT/DEN4 viruses in mice

Infecting virus		Mortality after i.p. inoculation	Mean of antibody titer (reciprocal) measured by		Mortality of survivors after i.p. inoculation with 100 or 1,000 i.p. LD_{50} of TP21	
Strain	Dose (PFU)		ELISA*	NT-test†	100	1,000
TP21	10^2	1/10	7,560	703	0/9	
E5	10^2	0/10	7,080	761	0/10	
DEN4	10^5	0/10	<50	<20	3/10	
TP21/DEN4	10^5	0/40	2,400	288	1‡/10	0/30
E5/DEN4	10^5	0/40	2,320	327	0/10	0/30
Control	NA	0/30	<50	<20	8§/10	17§/20

*Antibody titer in mouse serum collected 3 weeks after i.p. immunization was determined by using purified TP21 virions as antigen.
†Neutralizing antibodies were measured by a 50% plaque reduction neutralization test by using TP21 virus.
‡Traumatic death.
§Mice that survived i.p. challenge with TP21 were paralyzed for 3-5 days.

Immunogenicity and Protective Efficacy. Mice inoculated i.p. with 10^2 PFU of TP21 or E5 or 10^5 PFU of TP21/DEN4 or E5/DEN4 developed a high antibody response to TP21 virions (as measured by ELISA) (Table 4). In addition, these mice developed a high level of neutralizing antibodies against LGT TP21 as measured by plaque reduction. In contrast, mice inoculated i.p. with 10^5 PFU of DEN4 failed to develop TP21 neutralizing antibodies or TP21 antibodies measurable by ELISA.

Twenty-three days after inoculation with TP21/DEN4, E5/DEN4, DEN4, TP21, or E5, mice were challenged i.p. with 100 or 1,000 i.p. LD_{50} of TP21 (Table 4). All mice that had been immunized previously with TP21/DEN4 or E5/DEN4 were completely protected against i.p. challenge with TP21. In contrast, mice immunized i.p. with DEN4 were only partially protected: 3 of 10 mice died when challenged with 100 i.p. LD_{50} of LGT TP21. After challenge with TP21 each of the 30 nonimmunized control mice developed encephalitis and 25 subsequently died. As observed earlier, these results indicate that an immune response to DEN4 nonstructural proteins was not able to provide complete protection for mice against LGT TP21. In a subsequent study (Table 5), each of five mice inoculated with 10^5 PFU of DEN4 failed to resist challenge with 1,000 i.p. LD_{50} of LGT TP21, whereas 9 of 10 mice immunized i.p. with 10 PFU of chimeric TP21/DEN4 or E5/DEN4 survived the same LGT TP21 challenge. It appears that the LGT E and preM proteins represent the major protective antigens of the chimeras responsible for significant resistance to lethal i.p. LGT challenge.

It was possible that the immunogenicity and protective efficacy of the parental LGT viruses and their DEN4 chimeras resulted from immunization with preformed antigens present in viral preparations inoculated i.p. and not from antigens produced by virus that replicated *in vivo*. This issue was addressed by evaluating antibody responses and protective efficacy of virus preparations that contained only 10 PFU. In addition, the chimeras were evaluated at two other levels of infectivity, namely 10^3 or 10^5 PFU (Table 5). At each of the three doses evaluated, virus was tested without modification or after complete inactivation of infectivity by UV irradiation. Before performing this experiment the time required for complete inactivation of infectivity by UV was determined by kinetic analysis to be 60 min. As little as 10 PFU of either chimera regularly induced neutralizing and ELISA antibodies, whereas only 1 of 10 mice that received UV inactivated 10^5 PFU of a chimera developed a low level of ELISA antibodies and none developed measurable neutralizing antibodies. The response of these mice to i.p. challenge with TP21 (1,000 i.p. LD_{50}) was consistent with the immunological responses. Only 1 of 10 mice inoculated i.p. with 10 PFU of a chimera succumbed to challenge, whereas 9 of 10 mice that received UV inactivated 10^3 PFU and 7 of 10 that received UV inactivated 10^5 PFU died after challenge. It appears that successful immunization by the two chimeras primarily reflects the effect of viral replication and not the effect of a large mass of preformed viral antigens.

Table 5. Protective efficacy of live or inactivated chimeric LGT/DEN4 viruses tested in mice

Immunizing virus	Dose of immunization (PFU)	ELISA mean antibody titer	Mean neut. antibody titer	Mortality after i.p. challenge with 1,000 i.p. LD_{50} of TP21
E5/DEN4	10	240	52	0/5
	10^3	200	52	1/5
	10^5	1,530	151	0/5
E5/DEN4 (UV)	10*	<100	<20	4/5
	10^3*	<100	<20	4/5
	10^5*	100	<20	4/5
TP21/DEN4	10	240	65	1/5
	10^3	280	174	0/5
	10^5	3,680	257	0/5
TP21/DEN4 (UV)	10*	<100	<20	5/5
	10^3*	<100	<20	5/5
	10^5*	<100	<20	3/5
TP21	10	6,400	528	0/2
E5	10	6,400	536	0/3
DEN4	10^5	<100	<20	5/5
Control	NA	<100	<20	5/5

*Titer before UV irradiation that completely inactivated infectivity.

DISCUSSION

Our findings regarding the neurovirulence and neuroinvasiveness of LGT viruses are consistent with previous observations indicating that TP21 virus is more neurovirulent and significantly more neuroinvasive than its egg-passage derivative, strain E5 (8–10). Analysis and comparison of TP21 virus sequence with that of E5 revealed a limited number of amino acid differences in their polyprotein. The successful construction of viable LGT/DEN4 chimeras containing only preM and E genes of LGT origin should provide us with the opportunity to evaluate the contribution of each of the four mutations in the LGT insert to neurovirulence and neuroinvasiveness using site directed mutagenesis. Our failure to recover viable virus from certain LGT/DEN4 cDNAs that were chimeric with respect to nonstructural proteins allowed us to identify DEN4 and LGT protein incompatibilities than will be pursued more systematically in future studies. Finally, the prime incentive to create chimeras was to produce hybrid viruses that are: (*i*) infectious *in vivo*, (*ii*) free of detectable neuroinvasiveness, and (*iii*) yet able to induce protective immunity against viruses of the tick-borne flavivirus group.

Only 2 of the 16 LGT/DEN4 chimeric constructs were viable when RNA transcripts of full-length chimeric cDNA were tested

for infectivity in cell culture; these contained the genes for LGT preM and E. The chimeric TP21/DEN4 and E5/DEN4 viruses differed in LGT-derived preM and E sequences at only four amino acid positions in E. However, E5/DEN4 proved to be more restricted in growth in mosquito and simian cell lines and less cytopathic than TP21/DEN4. In addition, the E5/DEN4 chimera was found to be significantly less neurovirulent than the TP21/DEN4 chimera when assayed by IC inoculation of immunocompetent suckling mice. The magnitude of this difference was similar to that observed between the E5 and TP21 (Table 3), suggesting that one or more of the amino acid changes in the E protein of E5 played a major role in decreased neurovirulence.

Chimerization of LGT TP21 or E5 with DEN4 significantly reduced replicative capacity of the resulting virus in simian cells compared with either parental virus. As a consequence of this restriction, chimeric viruses could be recovered only when RNA transcripts of cDNA were transfected into mosquito cells. Virus isolated from transfected mosquito cells and then grown in these cells or simian cells failed to produce plaques in simian $LLCMK_2$ cells. In contrast, we previously observed that chimeric TBEV(ME)/DEN4 replicated efficiently in simian cells attaining a titer 1,000 times higher than DEN4 (11, 12).

In a previous study, chimerization of TBEV with DEN4 significantly reduced neurovirulence for mice (12). A similar large reduction of neurovirulence also was observed in the present study when LGT TP21 or E5 was chimerized with DEN4, suggesting that this might be a general phenomenon for viruses of the tick-borne flavivirus group. It appeared that the chimeric viruses retained the very low neurovirulence of their DEN4 parent rather than the higher mouse neurovirulence of their LGT virus parent.

During our previous studies, an antibody response to preM and E proteins of a TBEV(ME)/DEN4 chimera appeared to be sufficient to provide protection when mice were challenged i.p. with the highly virulent TBEV strain Sofjin (11) or when mice were challenged IC with a lethal dose of its chimeric virus (12). In the present study, we observed that 10 PFU of parental TP21 or E5 or 10^5 PFU of chimeric LGT/DEN4 virus was highly immunogenic when inoculated i.p. in Swiss mice, inducing a neutralizing antibody titer of 1:150 or higher (Tables 4 and 5).

SCID mice, which were deficient for immune functions mediated by T and B lymphocytes, proved to have a very sensitive system for detecting low levels of neuroinvasiveness of LGT TP21 or E5. Although SCID mice lack mature B and T lymphocytes, these animals do have normal innate immune functions, including functional macrophages, normal to elevated NK cell function, and elevated hemolytic complement activity (22, 25, 26). The ability of immunodeficient mice to detect neuroinvasiveness of TP21 or E5 was approximately 10^6 to 10^8 greater than was observed for immunocompetent Swiss mice (Table 3).

Our findings suggested that the parental TP21 virus or its attenuated derivative strain E5 might not to be suitable for use in human volunteers as a candidate live-attenuated vaccine against the TBE complex of viruses, as proposed earlier (5, 27, 28). The occurrence of vaccine-associated encephalitis, albeit at very low frequency ($10^{-4.3}$), which was observed during a large clinical trial of the attenuated LGT vaccine candidate (Yelantsev strain), supports this view (6, 7). Ideally, live attenuated tick-borne flavivirus vaccines should be free of this very low level of virulence. In contrast to parental LGT viruses, evaluation of TP21/DEN4 and E5/DEN4 chimeric viruses in SCID mice indicated that the two chimeras were free of any detectable neuroinvasiveness in this very sensitive assay system. As a consequence, the observation that a large dose (10^7 PFU) of either chimeric virus failed to invade the central nervous system and cause encephalitis or death after inoculation at a peripheral site is particularly reassuring. In addition, our findings suggest that proteins encoded by regions of the LGT genome other than the preM and E genes are required for either LGT virus to spread from a peripheral site to the brain and cause encephalitis.

The requisite balance between attenuation and immunogenicity of live virus vaccines is often difficult to achieve. In this instance, both LGT/DEN4 chimeras appear to be promising candidates because these viruses induced an asymptomatic infection in mice that provided effective protective immunity against challenge with 1,000 i.p. LD_{50} of wild-type LGT TP21. Also, in a previous study, a TBEV/DEN4 chimera was completely attenuated when inoculated by a peripheral route, but nonetheless, this chimera induced an antibody response that was associated with complete resistance to challenge by homotypic highly virulent TBEV (11). Based on our observations, these chimeric viruses as well as their more attenuated mutants appear to represent promising candidate TBEV vaccine strains. But before considering such LGT/DEN4 chimeras for use in a novel live TBEV vaccine, it remains to be determined whether immunization of mice or nonhuman primates with LGT/DEN4 chimeras also confers resistance to heterologous challenge with more virulent members of the TBE complex, such as the Far Eastern strains of TBEV. Earlier studies in animals and human volunteers suggest that this will be the case because immunization with attenuated LGT Tp21 or E5 induced a broad antibody response against various members of TBE complex (5, 6, 27, 28).

We are grateful to Dr. L.C. Iacono-Connors for providing LGT-specific hyperimmune mouse ascitic fluid and mAbs against LGT TP21 preM or E protein; Drs. J. Huggins and R. Shope for providing viruses; and D. Clark for oligonucleotide synthesis. Especially, we gratefully acknowledge the continued enthusiastic support of Drs. C.-J. Lai and R. Chanock.

1. Monath, T. P. & Heinz, F. X. (1996) in *Virology*, eds. Fields, B. N. & Knipe, D. M. (Raven, New York), pp. 961–1035.
2. Chambers, T. J., Hahn, C. S., Galler, R. & Rice, C. M. (1990) *Annu. Rev. Microbiol.* **44,** 649–688.
3. Pletnev, A. G., Yamshchikov, V. F. & Blinov, V. M. (1990) *Virology* **174,** 250–263.
4. Gordon Smith, C. E. (1956) *Nature (London)* **178,** 581–582.
5. Ilenko, V. I., Smorodincev, A. A., Prozorova, I. N. & Platonov, V. G. (1968) *Bull. W. H. O.* **39,** 425–431.
6. Smorodincev, A. A. & Dubov, A. V. (1986) in *Tick-Borne Encephalitis and Its Vaccine Prophylaxis*, ed. Smorodincev, A. A. (Meditsina, Leningrad), pp. 172–211.
7. Mandl, C. W., Iacono-Connors, L., Wallner, G., Holzmann, H., Kunz, C. & Heinz, F. (1991) *Virology* **185,** 891–895.
8. Thind, I. S. & Price, W. H. (1966) *Am. J. Epidemiol.* **84,** 193–213.
9. Thind, I. S. & Price, W. H. (1966) *Am. J. Epidemiol.* **84,** 214–224.
10. Nathanson, N., Thind, I. S., O'Leary, W. & Price, W. H. (1968) *Am. J. Epidemiol.* **88,** 103–111.
11. Pletnev, A. G., Bray, M., Huggins, J. & Lai, C.-J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10532–10536.
12. Pletnev, A. G., Bray, M. & Lai, C.-J. (1993) *J. Virol.* **67,** 4956–4963.
13. Mandl, C. W., Kunz, C. & Heinz, F. X. (1991) *J. Virol.* **65,** 4070–4077.
14. Dobrikova, E. Yu. & Pletnev, A. G. (1995) *Bioorg. Chem.* **21,** 528–534.
15. Wallner, G., Mandl, C. W., Kunz, C. & Heinz, F. X. (1995) *Virology* **213,** 169–178.
16. Iacono-Connors, L. C. & Schmaljohn, C. S. (1992) *Virology* **188,** 875–880.
17. Mandl, C. W., Heinz, F. X. & Kunz, C. (1988) *Virology* **166,** 197–205.
18. Mandl, C. W., Heinz, F. X., Puchhammer-Stockl, E. & Kunz, C. (1991) *BioTechniques* **10,** 485–486.
19. Cahour, A., Pletnev, A., Vazeille-Falcoz, M., Rosen, L. & Lai, C.-J. (1995) *Virology* **207,** 68–76.
20. Bray, M. & Lai, C.-J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10342–10346.
21. Iacono-Connors, L. C., Smith, J. F., Ksiazek, T. G., Kelley, C. L. & Schmaljohn, C. S. (1996) *Virus Res.* **43,** 125–136.
22. Bosma, G. C., Custer, R. P. & Bosma, M. J. (1983) *Nature (London)* **301,** 527–530.
23. Holzmann, H., Heinz, F. X., Mandl, C. W., Guirakhoo, F. & Kunz, C. (1990) *J. Virol.* **64,** 5156–5159.
24. Mandl, C. W., Guirakhoo, F., Holzmann, H., Heinz, F. X. & Kunz, C. (1989) *J. Virol.* **63,** 564–571.
25. Dorshkind, K., Pollack, S. B., Bosma, M. J. & Phillips, R. A. (1985) *J. Immunol.* **134,** 3798–3801.
26. Shultz, L. D., Schweitzer, P. A., Christianson, S. W., Gott, B., Schweitzer, I. B., *et al.* (1995) *J. Immunol.* **154,** 180–191.
27. Price, W. H., Thind, I. S., Teasdall, R. D. & O'Leary, W. (1970) *Bull. W. H. O.* **42,** 89–94.
28. Thind, I. S. & Price, W. H. (1966) *Am. J. Epidemiol.* **84,** 225–233.