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Evaluation of St. Louis encephalitis virus/dengue virus type 4 antigenic chimeric viruses in mice and rhesus monkeys

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Summary

To develop a live attenuated virus vaccine against St. Louis encephalitis virus (SLE), two antigenic chimeric viruses were generated by replacing the membrane precursor and envelope protein genes of dengue virus type 4 (DEN4) with those from SLE with or without a 30 nucleotide deletion in the DEN4 3′ untranslated region of the chimeric genome. Chimeric viruses were compared with parental wild-type SLE for level of neurovirulence and neuroinvasiveness in mice and for safety, immunogenicity, and protective efficacy in rhesus monkeys. The resulting viruses, SLE/DEN4 and SLE/DEN4Δ30, had greatly reduced neuroinvasiveness in immunodeficient mice but retained neurovirulence in suckling mice. Chimerization of SLE with DEN4 resulted in only moderate restriction in replication in rhesus monkeys, whereas the presence of the Δ30 mutation led to over-attenuation. Introduction of previously described attenuating paired charge-to-alanine mutations in the DEN4 NS5 protein of SLE/DEN4 reduced neurovirulence in mice and replication in rhesus monkeys. Two modified SLE/DEN4 viruses, SLE/DEN4-436,437 clone 41 and SLE/DEN4-654,655 clone 46, have significantly reduced neurovirulence in mice and conferred protective immunity in monkeys against SLE challenge. These viruses may be considered for use as SLE vaccine candidates and for use as diagnostic reagents with reduced virulence.

Keywords

St. Louis encephalitis virus; chimeric virus; vaccine

Introduction

St. Louis encephalitis virus (SLE), a mosquito-borne flavivirus, is a member of the Japanese encephalitis virus (JE) serocomplex, which also includes West Nile virus (WN) and Murray Valley encephalitis virus [1]. SLE was first isolated in 1933 from the brain of a deceased patient during an outbreak of approximately 1,000 cases of encephalitis in St. Louis Missouri [2]. More than 10,000 cases of severe disease have been described since then in the United States of America, and the virus remains endemic throughout most of the country. The most recent extensive outbreak of disease associated with SLE was in 1990 when over 200 cases with 14 deaths were reported in central Florida [3]. SLE is also endemic in Central and South America, and recent reports have indicated increased human disease associated with SLE in Brazil and Argentina [4,5]. Like WN, SLE is maintained in a natural transmission cycle between birds and *Culex* mosquitoes, and humans typically serve as incidental hosts [6]. High seroprevalence in sentinel chickens and positivity in mosquitoes during monitoring often predicts epidemic

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SLE human disease, as was the case in Florida in 1990. The ability to predict an increased period of SLE transmission indicates that a window of opportunity usually exists to vaccinate susceptible individuals. Human infection with SLE results in a spectrum of disease including asymptomatic infection, a general febrile illness, and potentially fatal meningitis/encephalitis [7]. The incidence of symptomatic to asymptomatic infection is reported to be approximately 1 to 300, although the elderly have a much greater risk of developing severe disease. Currently, a licensed vaccine is not available for prevention of SLE disease. The goal of the present study is to develop vaccine candidates for SLE that could be combined with our current WN vaccine candidate to form a bivalent vaccine that would provide protection from the two mosquito-borne flaviviruses that are endemic in the US.

Since 1999, WN has emerged as an important cause of encephalitis in humans and horses in North America. Outbreaks of febrile and neuroinvasive disease due to WN occur annually in the US, and a total of ~27,000 human cases have been identified between 1999 and 2007 (CDC Reports: http://www.cdc.gov/ncidod/dvbid/westnile). A live attenuated antigenic chimeric WN vaccine candidate, designated WN/DEN4Δ30, was generated by replacing the pre-membrane (prM) and envelope (E) protein genes of DEN4Δ30 with those of WN strain NY99 [8]. WN/DEN4Δ30 was found to be attenuated in mice, monkeys, geese, and mosquitoes [9-11]. Although the precise mechanisms of attenuation for WN/DEN4Δ30 are unclear, two genetic factors appear to contribute to the attenuation: (1) antigenic chimerization led to reduced neuroinvasiveness and neurovirulence in mice and restricted replication in monkeys and (2) the presence of the Δ30 deletion mutation in the 3′ untranslated region (UTR) at nucleotides 10,478-10,507 further attenuated WN/DEN4 for monkeys [9-11]. WN/DEN4Δ30 replicated to a peak titer of between $10^{3.4}$ and $10^{4.9}$ PFU/g of brain after intracerebral inoculation of suckling mice, whereas WN reached a peak titer of nearly 10^{12} PFU/g of brain indicating that the chimeric virus has greatly reduced neurovirulence. In rhesus monkeys, viremia was not detectable during WN/DEN4Δ30 infection, but a strong neutralizing antibody response was induced that conferred protection from wild type WN infection. Clinical evaluation of the WN/DEN4Δ30 vaccine candidate is in progress (ClinicalTrials.gov NCT00094718 and NCT00537147).

SLE and WN are closely related with 70-75% amino acid homology in the E glycoprotein, which is the main target of neutralizing antibodies, and evidence exists for some cross-protection between the two viruses and other members of the JE serocomplex in birds and hamsters [12,13]. However, a bivalent WN and SLE vaccine will likely be required to induce long-term immunity to both viruses. Generation of a bivalent vaccine for protection from WN and SLE offers several benefits over the pursuit of individual vaccines for each virus including ease of development, testing, and usage. Most importantly, the range of the two viruses overlaps in most areas of the USA and offering simultaneous vaccination against both of the relatively rare viruses would be advantageous in terms of economics and public health.

We sought to generate the live attenuated SLE component of such a bivalent vaccine using the approach described above that was successful in generating the WN candidate vaccine. SLE/DEN4 and SLE/DEN4Δ30 were generated by replacing the prM and E protein genes of DEN4 or DEN4Δ30 with those of the Hubbard strain of SLE. Antigenic chimerization of SLE with DEN4 or DEN4Δ30 resulted in greatly reduced neuroinvasiveness for severe combined immunodeficient (SCID) mice but no reduction in neurovirulence for immunocompetent mice. SLE/DEN4 was moderately attenuated and immunogenic in rhesus monkeys while SLE/DEN4Δ30 was over-attenuated. We sought to further attenuate the SLE/DEN4 antigenic chimeric virus by the introduction of paired charge-to-alanine attenuating mutations in the nonstructural NS5 protein of SLE/DEN4. The resulting SLE/DEN4 mutants exhibited reduced neurovirulence in mice and provided complete protection in rhesus monkeys against challenge with SLE.

Materials and methods

Cells and viruses

Vero cells (African green monkey kidney) were maintained in OptiPro SFM (serum-free medium) (Invitrogen, Grand Island, NY) supplemented with 4 mM L-glutamine (Invitrogen). SH-SY5Y cells (human neuroblastoma) were maintained in D-MEM/F-12 (Dulbecco's minimal essential medium) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, and 0.05 mg/ml gentamicin (Invitrogen). C6/36 cells (*Aedes albopictus* mosquito cells) were maintained at 32°C in MEM containing Earle's salts and 25 mM HEPES buffer (Invitrogen) and supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 mM non-essential amino acids (Invitrogen).

A mouse-brain-derived suspension of the SLE Hubbard strain was obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch, Galveston, TX. The SLE Hubbard strain was originally isolated from the brain of a deceased patient in Missouri in 1937. We selected SLE Hubbard strain as the parent virus in our studies because its E glycoprotein has a homology of nearly 99% with the consensus sequence of 60 strains of SLE isolated during a 1933 - 1998 period in North and South America from mosquitoes, mammals, or humans. For the present study, a virus stock was prepared in Vero cells that had a titer of 10^6 PFU/ml and is referred to here as uncloned SLE. Subsequently, the virus was biologically-cloned by two successive passages at terminal end-point dilution and finally amplified in Vero cells. This biologically-cloned SLE stock was used as a parental wild-type virus for animal studies and as a source of genomic RNA to prepare the cDNA of prM and E genes for chimeric virus constructions. Complete genome sequence analysis of uncloned SLE (GenBank accession no. EU566860) and the biologically-cloned SLE revealed two putative Vero cell-adaptation mutations: an Arg_{236}→Lys substitution located in the E protein and a Met_{153}→Val substitution in the NS4B nonstructural protein.

Construction and recovery of antigenic chimeric SLE/DEN4 viruses

The cDNA clones used to derive the SLE/DEN4 and SLE/DEN4Δ30 antigenic chimeric viruses were generated in a similar manner as was previously performed for the WN/DEN4 chimeric viruses (Figure 1A) [8]. The p4 plasmid (GenBank accession no. AY648301), that contains the full-length infectious cDNA for recombinant DEN4 (rDEN4), was used for the construction of chimeric SLE/DEN4. The source of the SLE cDNA was a PCR product that included nucleotides 408 to 2514 of the SLE genome. Two nucleotide (nt) changes (A > G at nt 1670 and T > C at nt 1700) were identified in the PCR fragment coding for the E protein, neither of which resulted in amino acid substitutions.

For construction of the SLE/DEN4 cDNA plasmid, a PCR fragment containing the SP6 promoter and the 5′ UTR and C gene of DEN4 was generated using forward primer 5′-TGACCATTTCCGGGCGCGCCACGGCGTTAC-3′ and reverse primer 5′-CAATGTTATACTAGTCCTTTTTCTCCCGTTCAA-3′ and was ligated into a modified polylinker region in pBR322. The forward primer contains an *Asc*I restriction site while the reverse primer contains a *Spe*I restriction site. The addition of the *Spe*I site altered the C/prM junction region in SLE by changing two amino acids in capsid, Gly_{104}→Thr and Gly_{105}→Ser. A PCR fragment containing the prM/E region (nt 416 - 2386) of SLE was generated using a forward primer (5′-AGAAAAAGGACTAGTGGCAGATCGTTGCTC-3′) that contains a *Spe*I restriction site sequence and a reverse primer (5′-GAGTCAGCGAGATGCTCCTGTCGCTCGAGTGCAACCCCATC-3′) that contains a *Xho*I site. This fragment was inserted into the modified pBR322 construct containing the SP6 promoter and the 5′UTR and C sequences of DEN4. Finally, the 5′UTR/C/prM/E fragment was inserted into p4 and p4Δ30 after digestion with *Asc*I and *Xho*I to generate SLE/DEN4 and

SLE/DEN4Δ30 that contain Thr-Ser-Gly-Arg (TSGR) at the P1′-P4′ positions of the C/prM proteolytic cleavage junction (Figure 1A). A second set of SLE/DEN4 and SLE/DEN4Δ30 plasmids was generated by site-directed mutagenesis and contain Gly-Gly-Thr-Arg (GGTR) at the C/prM junction (Figure 1A). The correct full-length chimeric virus genomes were confirmed by sequence analysis.

For recovery of viruses, 5′-capped RNA transcripts were synthesized *in vitro* from cDNA plasmids and transfected into either Vero cells or C6/36 cells. Briefly, plasmids were linearized with *Acc*65I and transcribed *in vitro* using SP6 polymerase. Purified transcripts were then transfected into Vero or C6/36 cells using DOTAP liposomes (Roche, Indianapolis, IN). Recovered viruses were amplified by passage in Vero cells and biologically-cloned by two or three terminal dilutions in Vero cells before experimental stocks were prepared. Titration of virus stocks was performed using a plaque assay in Vero cells with visualization of plaques by immunostaining with SLE-specific hyperimmune mouse ascitic fluid (ATCC, Manassas, VA).

Generation of SLE/DEN4 viruses with paired charge-to-alanine mutations

Eight paired charge-to-alanine mutations in the DEN4 NS5 gene that were previously described [14] were individually introduced into the SLE/DEN4 cDNA clone (Figure 1B). Fragments containing the desired paired charge-to-alanine mutation were excised from the previously constructed mutant p4 plasmids by restriction digest and introduced into the SLE/DEN4 cDNA clone containing the GGTR C/prM junction. Two sister plasmids were generated for each of the eight paired charge-to-alanine mutations for a total of 16 plasmids. Each plasmid was confirmed to have the correct paired charge-to-alanine mutation by sequence analysis. Viruses were recovered in C6/36 cells after transfection as described above and then passaged in Vero cells to reach a minimum virus titer of approximately 10^6 PFU/ml. Viruses were biologically-cloned by two or three terminal dilutions before experimental stocks were prepared in Vero cells. The resulting virus stocks were subjected to partial genome sequence analysis to confirm that the virus contained the SLE prM/E region and to determine if the correct paired charge-to-alanine mutation was present.

Studies in mice

All animal study protocols were approved by the NIAID Animal Care and Use Committee. Viruses were analyzed for neuroinvasiveness by intraperitoneal (IP) inoculation of 3-week-old, female immunocompetent Swiss Webster (SW) mice or SCID mice in groups of 5 or 10. SCID mice (*ICRSC-M*; Taconic, Germantown, NY) were administered ten-fold serial dilutions of virus in a 0.1 ml volume and were monitored daily for 49 days for signs of encephalitis. Encephalitis-specific clinical signs contributing to a moribund state in mice included tremors, seizure, prostration, and paralysis. To minimize pain or distress, moribund mice were euthanized immediately.

Neurovirulence of parental and chimeric viruses was assayed by intracerebral (IC) inoculation of 3-day-old SW mice (Taconic). Litters of approximately ten mice were inoculated with a 0.01 ml volume containing serial ten-fold dilutions of virus. Mice were monitored daily for 21 days for signs of encephalitis, and moribund mice were humanely euthanized.

For analysis of virus replication in the mouse brain, 5-day-old SW mice were inoculated IC with 10^3 PFU of SLE or a chimeric virus. The brains of four mice from each group were removed every other day from day 1 to 21 or until all mice from a group had succumbed to infection. Brains were individually homogenized to give a 10% suspension diluted in phosphate-buffered Hank's balanced salt solution (Invitrogen) supplemented with 7.5% sucrose, 5 mM sodium glutamate, 0.05 mg/ml ciprofloxacin, 0.06 mg/ml clindamycin, and 0.0025 mg/ml amphotericin. Brain suspensions were clarified by low-speed centrifugation and

frozen at -80°C. The virus titer in brain suspensions was determined by plaque assay in Vero cells.

Studies in rhesus monkeys

Studies in rhesus monkeys were conducted at Bioqual, Inc (Rockville, MD) following approval of the protocols by the ACUCs of both NIAID and Bioqual, Inc. Groups of rhesus monkeys (*Macaca mulatta*) were inoculated subcutaneously (SC) with 10^5 PFU of SLE or an antigenic chimeric virus. In one study, a ten-fold higher dose (10^6 PFU) of SLE/DEN4Δ30 was administered. Serum was collected for measurement of viremia on days 0-6, 8, and 10 and quantitated by plaque assay in Vero cells. Serum was drawn on day 28 to determine the levels of neutralizing antibody against SLE by plaque reduction neutralization test using wild type SLE (biologically cloned) or SLE/DEN4 as the target virus. Antibody titer was defined as the dilution of serum that neutralized 60% of virus. Neutralizing antibody titers against SLE and SLE/DEN4 were found to vary by two-fold or less in a subset of 10 SLE- or SLE/DEN4-inoculated rhesus monkeys (data not shown). All antibody titers reported in this study were determined using SLE/DEN4 so that assays could be performed under BSL-2 containment. Selected groups of mock and immunized animals were challenged SC with 10^5 PFU of SLE at day 35. Viremia was determined on days 0-6, 8, and 10 post-challenge.

Results

Recovery and sequence analysis of SLE/DEN4 and SLE/DEN4Δ30 viruses

Molecular cloning techniques were used to replace the prM/E region of the rDEN4 and rDEN4Δ30 viruses with the corresponding region of SLE to generate two viruses, SLE/DEN4 and SLE/DEN4Δ30, respectively (Figure 1A). Previous attempts to generate DEN4 antigenic chimeric viruses with tick-borne encephalitis virus (TBE), Langat, (LGT), and WN indicated that the sequence of the C/prM cleavage junction was important for viability [8, 15, 16]. Therefore, viruses with two different C/prM junctions were generated; GGTR and TSGR which represent amino acids in the P1′-P4′ position of the C/prM cleavage site (Figure 1A). Cleavage at this site is mediated by the viral NS2B/NS3 protease. The cDNA plasmid clone for each recombinant chimeric construct was designed to include one of two Vero cell adaptation mutations in the DEN4 NS4B gene (Thr_{105}→Ile or Leu_{112}→Phe) that were previously associated with enhanced replication of DEN4 parental or chimeric viruses in Vero cells [17]. SLE/DEN4 and SLE/DEN4Δ30 viruses with both GGTR and TSGR junctions were successfully recovered in either C6/36 cells or Vero cells followed by adaptation to Vero cell growth by serial passage and terminal dilution. Experimental stocks were then prepared in Vero cells, and each virus achieved titers of greater than 10^6 PFU/ml, which would permit the economical manufacture of a potential vaccine candidate.

Genomic sequence analysis was performed on each virus stock and the results are summarized in Table 1. The number of adventitious mutations that appeared in experimental virus stocks ranged from zero in SLE/DEN4 clone 551 to four mutations in SLE/DEN4 clone 549 and 554. The NS4B Thr_{105}→Ile and Leu_{112}→Phe mutations indicated in Table 1 were introduced into the cDNA clones to enhance recovery and replication in Vero cells, and are therefore not considered adventitious mutations. Two mutations in E (Ile_{70}→Thr and Phe_{156}→Ser) appeared in multiple viruses and may be adventitious mutations associated with increased replication in Vero cells.

Previously, a large panel of DEN4 viruses were generated that contained individual paired charge-to-alanine mutations in the NS5 polymerase gene and exhibited reduced replication in mouse brain, suggesting that the mutations might confer reduced neurovirulence [14]. Eight of the previously described paired charge-to-alanine mutations were selected for inclusion in

SLE/DEN4 (Figure 1B). Two virus clones of each modified SLE/DEN4 mutant virus were recovered (total of 16 viruses) in C6/36 cells and propagated in Vero cells. The SLE/DEN4-654,655 virus clones were found to be strongly temperature-sensitive (*ts*) in Vero cells at 37°C and were propagated at 32°C, while all other modified virus clones were successfully propagated at 37°C. Sequence analysis of the NS5 region containing the intended paired charge-to-alanine mutations was performed on the final experimental stock of each modified SLE/DEN4 virus. Thirteen of sixteen viruses contained the correct Ala-Ala sequence indicating the presence of the intended paired charge-to-alanine mutation. Three viruses contained sequence that did not match the plasmid sequence from which it was derived. SLE/DEN4-200,201 clone 52 contained an Ala codon at position 200 as designed, but a Val at position 201. SLE/DEN4-808,809 clone 47 contained an Ala codon at position 808, but a Glu at position 809. Finally, SLE/DEN4-808,809 clone 48 contained a Glu codon at position 808, and an Ala at position 809. Despite the presence of unintended coding changes in these three viruses, each of the sixteen modified SLE/DEN4 viruses were evaluated in mice. Full-length sequence analysis for three of the sixteen clones (41, 42, and 46) that had properties of interest upon subsequent evaluation was performed and nucleotide changes are indicated in Table 1.

Neuroinvasiveness and neurovirulence of SLE/DEN4 and SLE/DEN4Δ30 viruses in mice

First, the two parental SLE preparations, namely, uncloned SLE, which has only one passage in Vero cells, and biologically-cloned SLE were compared in suckling SW mice for neurovirulence following IC inoculation and in adult SW mice for neuroinvasiveness following IP inoculation. Neurovirulence is defined as the ability of virus to replicate in the CNS of suckling mice inoculated directly into the brain and cause fatal encephalitis, while neuroinvasiveness is defined as the ability of virus to replicate at a peripheral site and then spread to the CNS where encephalitis is produced. In side-by-side comparison of the LD_{50}, both uncloned and cloned SLE was (1) highly virulent for 3-day-old mice with an IC LD_{50} of 0.2 or 0.7 PFU, respectively, and (2) extremely neuroinvasive for 3-week-old SW mice with an IP LD_{50} of 32 or 5.6 PFU, respectively. These findings indicate that the differences in the sequences between these two preparations did not affect the highly virulent phenotype of SLE, and biologically-cloned SLE can be used as a reference parental virus for comparative study of neurovirulence and neuroinvasiveness of the newly generated chimeric SLE/DEN4 viruses in mice.

SLE/DEN4 and SLE/DEN4Δ30 were compared with biologically-cloned SLE for neuroinvasiveness and neurovirulence in mice. Neuroinvasiveness was assayed by IP inoculation of highly-sensitive SCID mice followed by daily monitoring for signs of encephalitis and moribundity. SLE was found to be highly neuroinvasive for adult SCID mice with an IP LD_{50} of 3.2 PFU (Table 2). With the exception of the SLE/DEN4 clone 549, the two other SLE/DEN4 clones and the three SLE/DEN4Δ30 clones were found to be restricted for mouse neuroinvasiveness with at least a 30,000- to 300,000-fold increase in LD_{50} when compared to SLE. Infrequent paralysis or death (from 10 to 20%) was observed in the permissive SCID mice inoculated with SLE/DEN4 clone 551 and 554 and SLE/DEN4Δ30 clone 545. In addition, the average survival time of mice, which succumbed to infection, was typically three times longer for chimeric virus-inoculated animals than for animals inoculated with SLE (data not shown). Since the SLE/DEN4 viruses were so strongly attenuated, it appears that chimerization was responsible in large part for the attenuation and the contribution of the Δ30 mutation was not required for reduced neuroinvasiveness. The presence of a GGTR or TSGR C/prM junction did not appear to influence the level of neuroinvasiveness of the tested viruses. One of the six chimeric viruses tested, SLE/DEN4 clone 549, did not appear to have the high degree of reduced neuroinvasiveness observed for the other chimeric viruses ($LD_{50} < 10^4$ PFU). However, deceased mice inoculated with SLE/DEN4 clone 549 did have a four-fold increase in average survival time when compared to SLE (data not shown). The reason

for the increased neuroinvasiveness of clone 549 relative to the other SLE/DEN4 and SLE/DEN4Δ30 viruses may be due to one or more of the adventitious mutations present in the virus.

Neurovirulence was measured by IC inoculation of SW suckling mice and daily monitoring. Biologically-cloned SLE was found to be highly neurovirulent in suckling mice with an IC LD_{50} of 0.4 PFU (Table 2). Four viruses (SLE/DEN4 clone 551 and SLE/DEN4Δ30 clones 545, 546, and 609) had LD_{50} values that were only 5- to 10-fold different than that of SLE. Therefore, in contrast to the effect of chimerization on the attenuation of neuroinvasiveness, chimerization of SLE with DEN4 did not substantially reduce neurovirulence and the presence of the Δ30 also had no effect. Interestingly, two SLE/DEN4 viruses, clone 549 and clone 554, had greatly reduced neurovirulence; their LD_{50} values were at least 250-fold and 2,500-fold higher than observed for SLE, respectively. These two viruses share a common mutation (Phe_{156}→Ser) in the E glycoprotein that was not observed in the other four viruses tested, and it is possible that this coding change may confer the reduced neurovirulence (Table 1). Again, the presence of the GGTR or TSGR C/prM junction in the chimeric viruses did not appear to influence neurovirulence. SLE/DEN4 viruses with the GGTR sequence were arbitrarily chosen for studies in rhesus monkeys described below.

Neuroinvasiveness and neurovirulence of SLE/DEN4 viruses bearing charge-to-alanine mutations in mice

Since the SLE/DEN4 viruses retained a high level of neurovirulence for mice, we sought to further attenuate the virus by the introduction of charge-to-alanine mutations that were previously shown to attenuate DEN4 virus for replication in mouse brain (Figure 1B). An initial experiment was performed to screen the 16 modified SLE/DEN4 viruses for reduced neurovirulence in mice (Figure 2). Groups of SW suckling mice were inoculated IC with 10^2 PFU of each virus and compared to biologically-cloned SLE and SLE/DEN4 clone 551. As expected, both SLE and SLE/DEN4 were almost uniformly lethal at this dose. Infection with three of the modified SLE/DEN4 viruses resulted in a substantially greater survival rate. Survival rates were 78% and 100% for mice inoculated with SLE/DEN4-654,655 clone 45 or clone 46, respectively. In the case of SLE/DEN4-436,437, clone 41 was highly attenuated, while all mice inoculated with clone 42 succumbed to infection which suggested that a genetic difference other than the NS5 $Asp_{436}Lys_{437}$→AlaAla mutation was likely responsible for the reduced virulence. Complete genomic sequence analysis of clone 41 indicated the presence of an Ala_{63}→Ser substitution and the previously identified Phe_{156}→Ser mutation in E, which was also associated with the reduced neurovirulence observed in SLE/DEN4 clones 549 and 554 (Table 2). The Phe_{156}→Ser mutation in E was not found in any of the other 15 modified SLE/DEN4 viruses. Therefore, based on the association of the presence of the E Phe_{156}→Ser mutation with reduced neurovirulence in 3 independent SLE/DEN4 viruses, it is likely that this amino acid change is responsible for the phenotype.

Based on these initial results, SLE/DEN4-436,437 clone 41 and SLE/DEN4-654,655 clone 46 were further evaluated for neuroinvasiveness and neurovirulence by determination of LD_{50} values in mice (Table 2). Like the parental SLE/DEN4 clone 551, tche modified viruses had reduced neuroinvasiveness at the highest dose tested in adult SCID mice. In addition, the LD_{50} values for IC inoculated suckling mice for SLE/DEN4-436,437 clone 41 ($>10^3$ PFU) and SLE/DEN4-654,655 clone 46 (428 PFU) confirmed that these two viruses were significantly attenuated for neurovirulence compared to both SLE and SLE/DEN4.

We next sought to quantitate the level of virus replication in mouse brain of SLE, SLE/DEN4 clone 551, and the further attenuated derivatives, SLE/DEN4-436,437 clone 41 and SLE/DEN4-654,655 clone 46. Similar to previous studies of WN, wild-type SLE rapidly reached an extremely high mean peak virus titer in the brain ($10^{10.1}$ PFU/g) after IC inoculation (Figure 3) [10]. SLE/DEN4 clone 551 did not appear to be attenuated and reached a mean peak virus

titer in the brain of $10^{9.3}$ PFU/g which was not surprising based on the nearly wild-type level of neurovirulence previously observed (Table 2). SLE/DEN4-654,655 clone 46 demonstrated a delay in replication compared to SLE and SLE/DEN4 and reached a mean peak virus titer in the brain of $10^{7.2}$ PFU/g, an approximately 1,000-fold reduction from SLE indicating a significant level of attenuation for replication in the mouse brain. Even more striking, SLE/DEN4-436,437 clone 41 only reached a mean virus titer of $10^{4.5}$ PFU/g, which represents a nearly 400,000-fold reduction in replication compared to SLE.

Replication and immunogenicity in rhesus monkeys

Two antigenic chimeric viruses, SLE/DEN4 clone 551 and SLE/DEN4Δ30 clone 545, were first selected for study in rhesus monkeys because they contained minimal adventitious mutations, which would enable an accurate assessment of the contribution of chimerization and the Δ30 mutation to replication and immunogenicity in rhesus monkeys. These two viruses also exhibited greatly reduced neuroinvasiveness compared to wild type SLE. SLE/DEN4 clone 551 contains no adventitious mutations, while SLE/DEN4Δ30 clone 545 contains a coding change in prM, Lys_{46}→Glu (Table 1), which is the only change between the two viruses other than the Δ30 mutation.

Nine of ten monkeys inoculated with biologically-cloned SLE became viremic, and the virus was found to replicate to a mean peak virus titer of $10^{2.1}$ PFU/ml with a mean number of 3.5 viremic days (Table 3). The mean serum neutralizing antibody titer was 1:39 and is lower than reported for other flaviviruses [9,18-21]. Five of six monkeys inoculated with SLE/DEN4 developed viremia with a mean duration of 2.2 days. The mean peak virus titer ($10^{1.1}$ PFU/ml) was significantly lower than the peak SLE titer ($10^{2.1}$ PFU/ml) ($P < 0.05$). Despite the reduced replication of SLE/DEN4, the mean serum neutralizing antibody titer (1:109) was robust and comparable to antibody levels induced by SLE. In contrast, SLE/DEN4Δ30 was found to be over-attenuated in rhesus monkeys as no monkeys developed detectable viremia, and neutralizing antibodies were not detected. In a separate experiment, four monkeys were inoculated with a ten-fold higher dose of SLE/DEN4Δ30 (10^6 PFU) and were also found to not develop viremia or sufficient neutralizing antibody levels indicating that the Δ30 mutation confers strong attenuation or reduced infectivity upon SLE/DEN4 in monkeys (data not shown).

Based on the reduced mouse neurovirulence and restricted replication in mouse brain of SLE/DEN4-436,437 clone 41 and SLE/DEN4-654,655 clone 46, these two viruses were next evaluated for replication and immunogenicity in rhesus monkeys in a comparative study including SLE and SLE/DEN4 clone 551, and the cumulative data is shown in Table 3. Three of four monkeys immunized with SLE/DEN4-436,437 clone 41 developed viremia, and the mean number days of viremia (2.0 days) and mean peak virus titer ($10^{1.0}$ PFU/ml) were similar to the levels observed in SLE/DEN4-immunized animals. The mean peak virus titer was significantly lower than that of SLE ($P < 0.05$). The antibody response (1:28) induced by SLE/DEN4-436,437 was also comparable to that observed for animals immunized with SLE (1:39) or SLE/DEN4 (1:109). These results indicate that the addition of either the NS5 $Asp_{436}Lys_{437}$→AlaAla mutation or the E Phe_{156}→Ser mutation in SLE/DEN4 does not confer further attenuation in rhesus monkeys beyond the level conferred by antigenic chimerization. In contrast, the monkeys inoculated with SLE/DEN4-654,655 clone 46 had no detectable viremia and no monkey seroconverted to SLE although weak antibody responses were detected. These results suggest that the presence of the NS5 $Asp_{654}Arg_{655}$→AlaAla mutation in SLE/DEN4 had a potentially over-attenuating effect in rhesus monkeys similar to that observed by the Δ30 mutation in SLE/DEN4Δ30.

Groups of monkeys inoculated with SLE, SLE/DEN4, or SLE/DEN4Δ30 (10^5 PFU dose only) were challenged with 10^5 PFU of SLE on day 35 after immunization. As expected based on

the observed neutralizing antibody responses, SLE- and SLE-DEN4-immunized animals were completely protected from the development of viremia. In contrast, three of four monkeys immunized with SLE/DEN4Δ30 developed viremia after challenge with SLE although the mean duration (1.0 day) and mean peak virus titer ($10^{1.1}$ PFU/ml) was lower than in mock-immunized monkeys challenged with SLE.

The groups of four rhesus monkeys inoculated with SLE/DEN4-436,437 and SLE/DEN4-654,655 were also challenged with SLE on day 35 post-infection. Each animal was protected as demonstrated by a lack of detectable viremia. This was not surprising based on the immunogenicity of SLE/DEN4-436,437, but was somewhat unexpected for monkeys immunized with SLE/DEN4-654,655 since the pre-challenge SLE-specific antibody levels were lower. However, in contrast to the SLE/DEN4Δ30-immunized animals, which had no detectable antibody and were not fully protected from SLE challenge, each of the four SLE/DEN4-654,655-immunized animals had at least a detectable neutralizing antibody titer against SLE (data not shown).

Evaluation of the temperature sensitivity of SLE/DEN4 viruses

To determine if the lack of detectable replication and decreased immunogenicity of SLE/DEN4-654,655 was due to temperature sensitivity of virus replication, the modified SLE/DEN4 viruses were analyzed for plaque formation in Vero cells and SH-SY5Y human neuroblastoma cells at varying temperatures. As mentioned above, both SLE/DEN4-654,655 clones (45 and 46) were found to be *ts* upon passage in Vero cells and was propagated at 32° C. SLE and SLE/DEN4 were shown to replicate efficiently at 39°C (Figure 4). SLE/DEN4-436,437 clone 41 was found to be moderately *ts* at 39°C when compared to permissive temperature, 32°C. In contrast, plaque formation of SLE/DEN4-654,655 was reduced at 37°C and completely abrogated at 38°C in both cell types. These results indicate that a strong *ts* phenotype associated with the NS5 654,655 paired charge-to-alanine mutation may be a factor in the over-attenuation observed in rhesus monkeys. However, temperature sensitivity did not account for the over-attenuation of SLE/DEN4Δ30 in rhesus monkeys, since this virus was not *ts* at temperatures up to 39°C (data not shown).

Discussion

Since the first reports of the generation of intratypic (DEN2/DEN4) and intertypic (TBE/DEN4) antigenic chimeric flaviviruses using reverse genetics [15,22], numerous chimeric viruses have been created using genes from tick-borne and mosquito-borne flaviviruses, and many of these viruses are currently being evaluated as vaccines [10,16,19,20,23-26]. Recent clinical studies have indicated that antigenic chimeric flaviviruses are attenuated and immunogenic in human volunteers and may serve as live attenuated virus vaccines for protection against disease caused by DEN, JE, WN, and TBE viruses [27-31]. These positive results observed in several clinical trials support the further development and study of antigenic chimeric flaviviruses.

Attenuation of antigenic chimeric flaviviruses for mice, mosquitoes, or non-human primates can result from two different mechanisms. First, the simple construction of an antigenic chimeric virus from two wild-type parent viruses can yield a virus that has either reduced virulence or a restricted level of replication compared with either parent virus. For example, the DEN2/DEN4 and the DEN3/DEN4 chimeric viruses were found to be attenuated in rhesus monkeys and mosquitoes without the introduction of any specific attenuating mutations [20, 32]. The WN/DEN4 and the LGT/DEN4 chimeric viruses were also attenuated for neurovirulence and neuroinvasiveness for mice and for replication in monkeys [8-10,16,21]. In these examples, antigenic chimerization led to attenuation of both neuroinvasiveness and neurovirulence in mice for the neurotropic viruses or to restricted replication in rhesus monkeys

for each of the four viruses. However, the TBE/DEN4 chimeric virus exhibited greatly reduced neuroinvasiveness but retained a high level of neurovirulence for mice indicating that chimerization in this case did not result in a decrease in virulence for the murine CNS [19, 33]. In addition, the TBE/DEN4 chimeric virus replicated efficiently in rhesus monkeys [19]. In the present study, we had expected that the chimerization of SLE with DEN4 would result in a pattern of neuroinvasiveness and neurovirulence in mice and replication in monkeys that was similar to the chimerization of its closest relative, WN, but this did not turn out to be the case. Rather, SLE/DEN4 resembled the TBE/DEN4 chimeric virus exhibiting diminished neuroinvasiveness while retaining a high level of neurovirulence in mice. SLE/DEN4, like TBE/DEN4, exhibited only moderate attenuation in rhesus monkeys. These results indicate that antigenic chimerization routinely yields vaccine candidates that have restricted neuroinvasiveness for mice, but does not always yield chimeric viruses with a predictable level of mouse neurovirulence or restricted replication in rhesus monkeys. Thus, the mechanism of the attenuation afforded by the incompatibility of antigenic chimeric virus proteins appears to be governed by highly virus-specific genetic elements whose in vivo effects cannot be predicted by genetic or antigenic relatedness. The molecular mechanisms underlying these strain specific differences remain completely unknown.

The second mechanism that leads to attenuation of antigenic chimeric viruses is the presence of attenuating mutations either in the genetic background of the antigenic chimeric virus [19, 34] or in the prM or E protein [33,35]. Several attenuated flaviviruses have been utilized to generate vaccine candidates including the yellow fever vaccine virus [35], a DEN2 virus that has been attenuated by serial in vitro passage [36], and, in the present study, a DEN4 virus attenuated by a deletion (the Δ30 deletion mutation) in the 3′UTR [37]. The Δ30 deletion mutation in the DEN4 virus has been shown to be highly attenuating and genetically stable in humans and, as such, has been selected for inclusion in antigenic chimeric viruses [38]. Addition of the Δ30 deletion mutation to the DEN2/DEN4 [20] and the DEN3/DEN4 [32] chimeric viruses did not further attenuate the virus for rhesus monkeys, but the mutation had a highly significant attenuating effect on TBE/DEN4 [19] and WN/DEN4 [9,10] in rhesus monkeys. In the present study, addition of the Δ30 deletion mutation to SLE/DEN4 over-attenuated the virus for rhesus monkeys resulting in no detectable serum neutralizing antibodies after immunization and insufficient protection from SLE challenge. Thus, the Δ30 deletion mutation was highly attenuating in both WN/DEN4 and SLE/DEN4, but the level of attenuation for SLE/DEN4 was sufficiently high that it rendered the chimeric virus poorly immunogenic in rhesus monkeys, and, therefore it was not useful as a vaccine for SLE. A set of at least three properties that identify an antigenic chimeric flavivirus as suitable for evaluation in humans include: (1) evidence of decreased viremia in rhesus monkeys; (2) ability to induce a protective immune response in monkeys; and (3) reduced neurovirulence in mice since some viruses that exhibit significant neurovirulence for mice can retain neurovirulence for the CNS of primates [39].

Since SLE/DEN4 was only moderately attenuated and SLE/DEN4Δ30 was over-attenuated in rhesus monkeys and both viruses retained neurovirulence for mice, we sought to identify additional mutations that could further attenuate SLE/DEN4. To potentially achieve a reduction in the neurovirulence of SLE/DEN4 for mice, paired charge-to-alanine mutations that were previously shown to reduce replication of rDEN4 in the mouse brain were introduced into the SLE/DEN4 chimeric virus. Two mutations, NS5 $Asp_{654}Arg_{655}$→AlaAla and E Phe_{156}→Ser, were identified that conferred one or more of the three desirable properties outlined above. The NS5 $Asp_{654}Arg_{655}$→AlaAla mutation, which has a strong *ts* phenotype, conferred reduced neurovirulence and reduced replication in the brain of suckling mice inoculated with SLE/DEN4-654,655. SLE/DEN4-654,655 manifested an approximately 1,000-fold reduction in peak virus titer and an eight day delay in attaining peak virus titer when compared with mice infected with SLE. However, this reduction was less than that exhibited by WN/DEN4Δ30

[10]. Importantly, SLE/DEN4-654,655 was highly restricted in replication in rhesus monkeys as no monkey had detectable viremia, indicating that the introduction of the 654,655 mutation into SLE/DEN4 further attenuated this virus for both rhesus monkeys and for the CNS of mice. Although SLE/DEN4-654,655 was only weakly immunogenic in rhesus monkeys, immunization with it provided complete protection against replication of SLE challenge virus in the monkeys. Thus, this mutation achieved each of the three desired properties of an acceptable SLE vaccine candidate. Since SLE/DEN4-654,655 is a temperature sensitive virus and since rhesus monkeys have a higher core body temperature (39°C) than humans (37°C), it is possible that SLE/DEN4-654,655 will replicate to a greater extent in humans than in rhesus monkeys and thereby will induce a higher level of neutralizing antibodies and potentially greater reactogenicity in the human host. The NS5 $Asp_{654}Arg_{655}$→AlaAla mutation contains 4 nucleotide substitutions and is expected to exhibit greater genetic and phenotypic stability than that of a virus with a single point mutation, but this requires experimental verification.

An adventitious mutation, Phe_{156}→Ser in the E glycoprotein, developed independently in three SLE/DEN4 chimeric viruses, each of which manifested greatly reduced neurovirulence in mice. The association of the mutation in three separate viruses with reduced neurovirulence in mice suggests a causal relationship. SLE/DEN4-436,437 clone 41, which contained this mutation, was highly attenuated and greatly restricted in replication in the CNS of mice whereas another clone of this virus lacking the Phe_{156}→Ser mutation retained neurovirulence. SLE/DEN4-436,437 clone 41 exhibited over a 400,000-fold reduction in peak titer compared to that of SLE. However, SLE/DEN4-436,437 clone 41 was not more attenuated than SLE/DEN4 in rhesus monkeys. As expected, it was immunogenic and provided protection against challenge. The reduced neurovirulence presumably mediated by Phe_{156}→Ser in E is conferred by a single nucleotide substitution and therefore may be susceptible to genetic instability or reversion. Reliance on a single nucleotide substitution to confer this reduction of neurovirulence may limit the utility of this vaccine candidate at present. Clearly, the genetic stability and potential for reversion of these viruses will need to be studied further. We are currently constructing a recombinant virus with an engineered E Phe_{156}→Ser mutation to confirm the in vivo effects of this mutation.

Since the E Phe_{156}→Ser mutation was not present in the cDNA clones used to recover each of the three SLE/DEN viruses (clones 549, 554, and 41), it is possible that the chimeric viruses bearing this adventitious mutation replicate more efficiently in Vero cells and were independently selected during the cloning process. Analysis of the E Phe_{156}→Ser locus indicated that this amino acid change would result in a putative N-linked glycosylation site at amino acids 154-156 (Asn-Tyr-Phe→Asn-Tyr-Ser) that is present in other SLE virus strains [40]. In the present study, we have not investigated the glycosylation state of the E protein of the chimeric viruses with this change, but such studies will be performed on the recombinant virus that is being engineered to contain only the E Phe_{156}→Ser mutation. SLE strains have been identified with varying patterns of E glycosylation, but the contribution of the carbohydrate chain at the glycosylation site to virulence is not well-defined [40,41] and needs further study.

Current studies focus on the identification of a modified SLE/DEN4 vaccine candidate that exhibits restricted neurovirulence in mice and low to undetectable viremia in rhesus monkeys while maintaining the ability to induce a protective immune response. Once a suitably attenuated and immunogenic vaccine candidate for SLE is found, a bivalent vaccine will be prepared with our current WN vaccine candidate, WN/DEN4Δ30, and tested in rhesus monkeys. Such a bivalent vaccine would provide protection from the two neurotropic, mosquito-borne flaviviruses endemic in the United States and could be used for at-risk groups including the elderly. This would serve as an economically feasible way to protect humans from these two virus infections, which can cause severe neurological manifestations. In

addition, one or more of these attenuated viruses could be considered for use as a diagnostic reagent at biosafety level 2.

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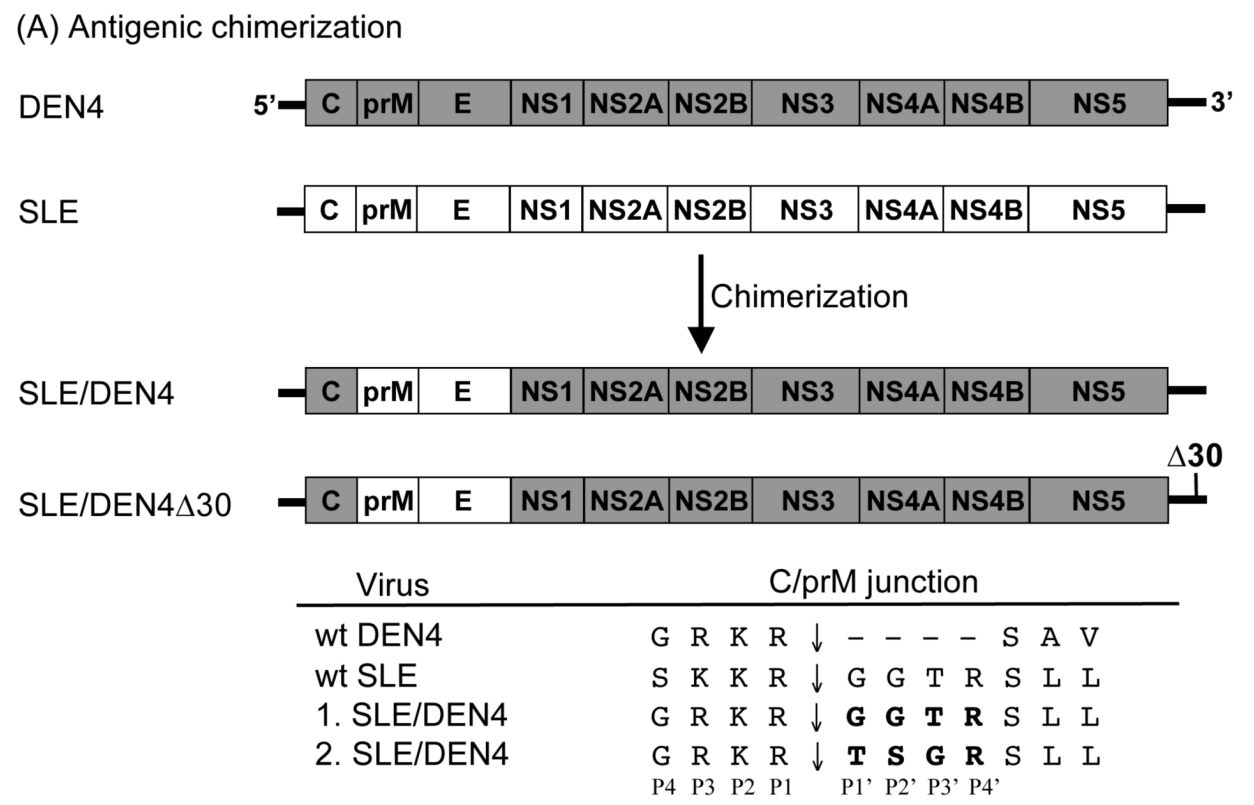

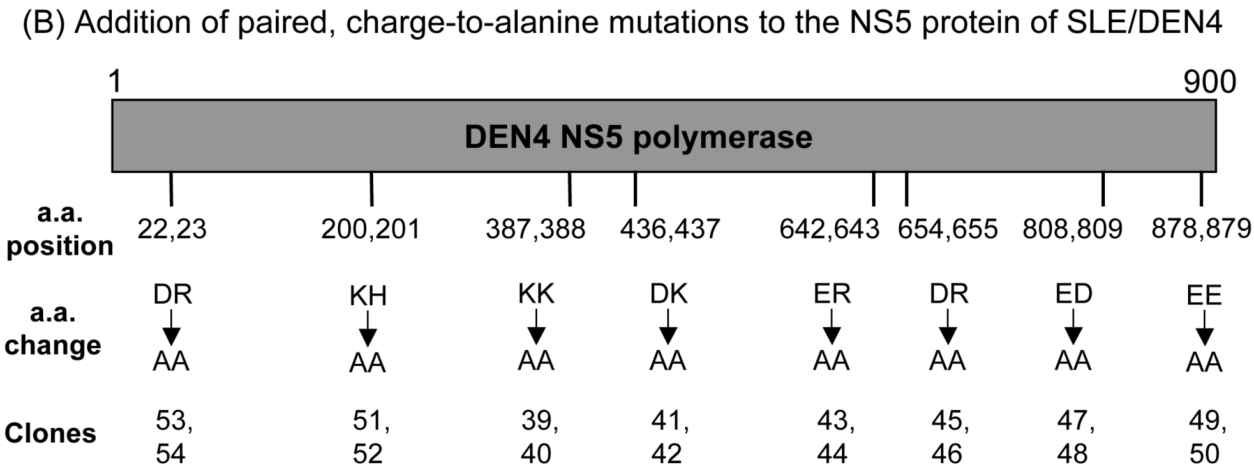

Figure 1. Molecular construction of recombinant SLE/DEN4 antigenic chimeric viruses

(A) The prM/E structural protein region of the DEN4 cDNA plasmid p4 [37] was replaced with the corresponding region from SLE Hubbard to generate the SLE/DEN4 virus. The Δ30 deletion, a 30 nucleotide deletion in the 3′UTR (DEN4 nucleotides 10,478-10,507), was introduced into the SLE/DEN4 cDNA plasmid and used to recover the SLE/DEN4Δ30 virus. The amino acid sequences surrounding the C/prM junctions at the protease cleavage site of parental and chimeric viruses are indicated. The P1′-P4′ amino acids of the resulting C/prM cleavage junctions in the SLE/DEN4 viruses are indicated in bold font. Separate SLE/DEN4 and SLE/DEN4Δ30 viruses were generated with both the GGTR or TSGR junctions. Arrows indicate the putative cleavage site at the C/prM junction mediated by the NS2B-NS3 protease.

(B) The eight pairs of charge-to-alanine mutations introduced into the DEN4 NS5 protein in SLE/DEN4 are indicated. The numbering indicates the position of the amino acid (a.a.) pair within the NS5 protein. Each individual wild type pair of amino acids was mutated to a pair of alanines in the SLE/DEN4 cDNA clone to generate eight modified SLE/DEN4 viruses. Two independent virus clones were recovered for each mutation and are identified by a 3 digit clone number; e.g. SLE/DEN4-22,23 clones 53 and 54.

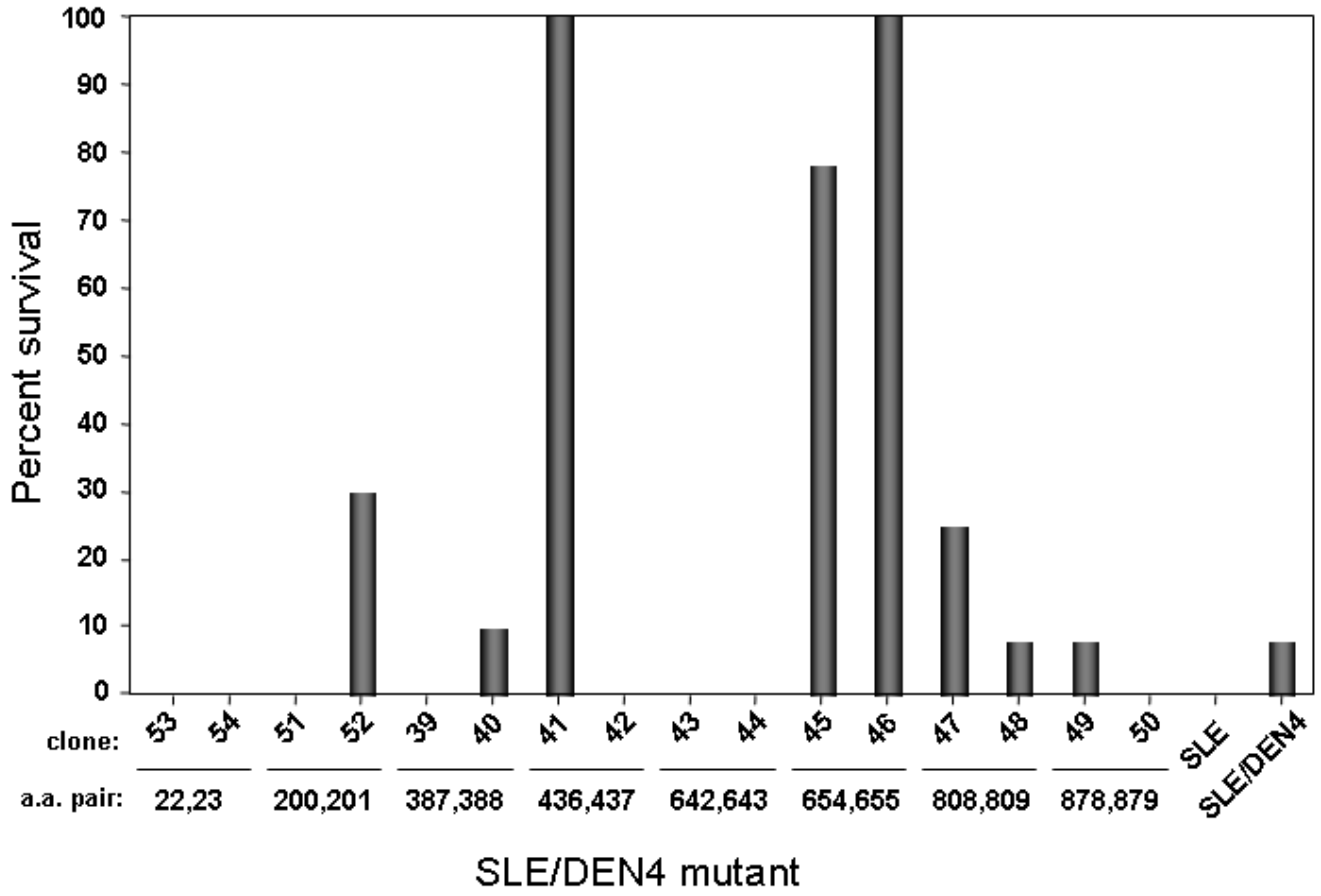

Figure 2. Neurovirulence of modified SLE/DEN4 viruses

Litters of approximately ten three-day-old Swiss Webster mice were inoculated IC with 10^2 PFU of indicated virus. Mice were monitored for signs of encephalitis and morbidity for 21 days and the percent survival for each group is indicated. Two virus clones for each modified SLE/DEN4 virus were included in the study.

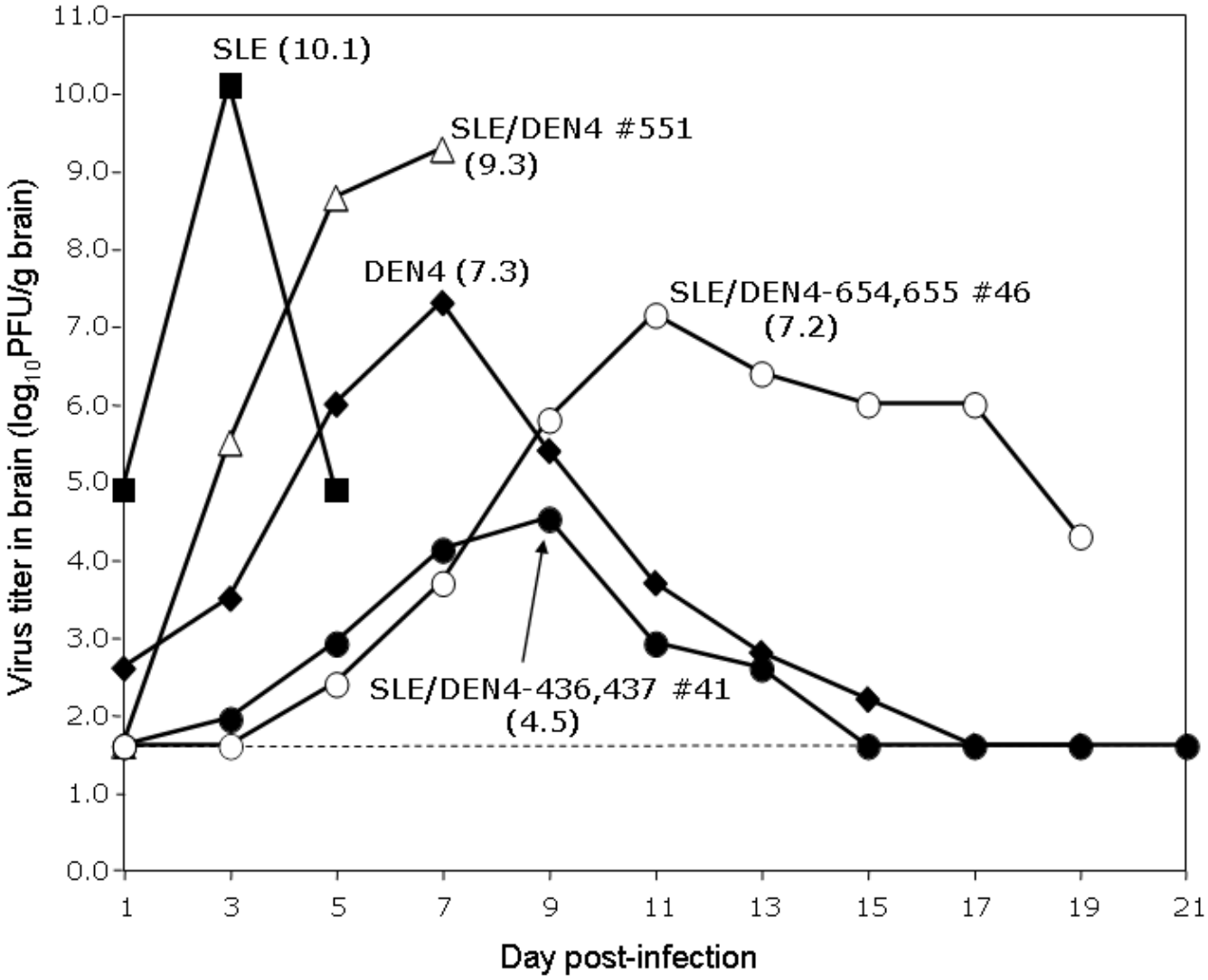

Figure 3. Replication of parental and chimeric viruses in mouse brain

Five-day-old Swiss Webster mice were inoculated IC with 10^3 PFU of indicated virus. Brains were removed on odd days post-infection from four mice per group and virus titer was determined by plaque assay in Vero cells. Mean peak virus titers (log_{10}PFU/g of brain) are indicated in parentheses. The limit of detection ($10^{1.7}$ PFU/g) is indicated by a dashed line. The end of a data line prior to day 21 indicates that all mice had succumbed to infection. The values for DEN4 are historical data included for comparison [10].

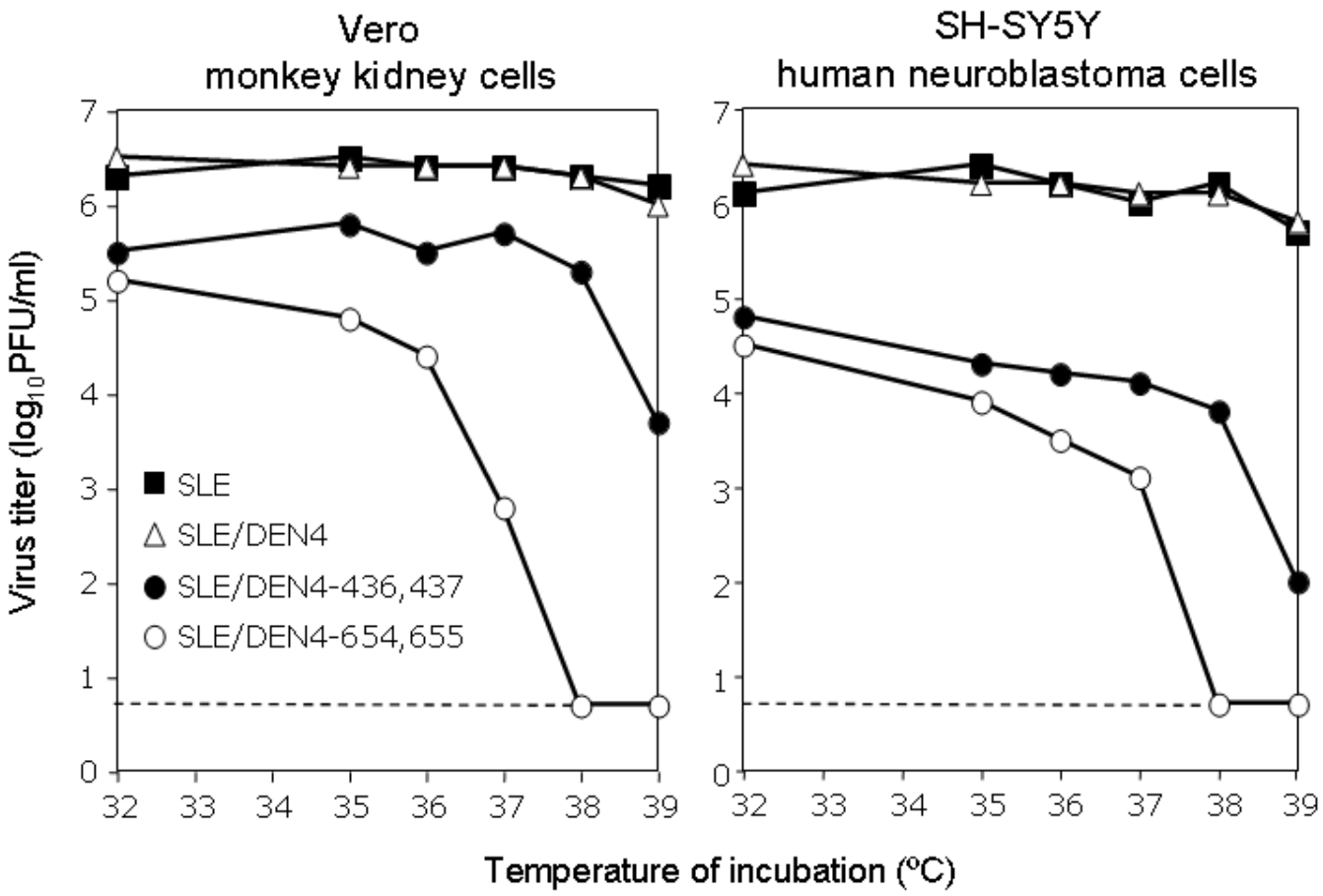

Figure 4. Growth analysis of parental SLE and chimeric viruses in Vero cells or in human neuroblastoma cells following incubation at different temperatures

An efficiency of plaque formation assay was performed with the indicated viruses in Vero cells and SH-SY5Y cells. Confluent monolayers of cells were infected with serial ten-fold dilutions of virus at 32°C, overlaid with semisolid growth media, and then incubated for five days at 32, 35, 36, 37, 38, or 39°C. Plaques were visualized by immunostaining and quantitated. The limit of detection ($10^{0.7}$ PFU/ml) is indicated by a dashed line.

Table 1

Mutations identified after Vero cell passage of SLE/DEN4 viruses

Virus	C/prM junction	Clone	Gene	Nucleotide position	Nucleotide substitution	Amino acid change[a]
SLE/DEN4	GGTR	549	E	1162	U→C	Ile_{70}→Thr
			E	1420	U→C	Phe_{156}→Ser
			E	1506	A→G	Thr_{185}→Ala
			NS4B	**7196**[b]	**A→C**	**Leu_{112}→Phe**
			3′ UTR	10341	U→C	-
SLE/DEN4	GGTR	551	**NS4B**	**7196**[b]	**A→C**	**Leu_{112}→Phe**
SLE/DEN4	TSGR	554	E	1162	U→C	Ile_{70}→Thr
			E	1362	U→C	Tyr_{137}→His
			E	1420	U→C	Phe_{156}→Ser
			NS3	4710	G→A	Val_{52}→Met
			NS4B	**7174**[b]	**C→U**	**Thr_{105}→Ile**
SLE/DEN4Δ30	GGTR	545	prM	588	A→G	Lys_{46}→Glu
			NS4B	**7196**[b]	**A→C**	**Leu_{112}→Phe**
SLE/DEN4Δ30	GGTR	546	E	1162	U→C	Ile_{70}→Thr
			NS1	2827	U→C	Phe_{124}→Ser
			NS3	5857	U→C	Leu_{434}→Pro
			NS4B	**7196** [b]	**A→C**	**Leu_{112}→Phe**
SLE/DEN4Δ30	TSGR	609	E	1162	U→C	Ile_{70}→Thr
			NS3	4879	A→C	His_{108}→Pro
			NS4B	**7174** [b]	**C→U**	**Thr_{105}→Ile**
SLE/DEN4-436,437	GGTR	41	E	1140	G→U	Ala_{63}→Ser
			E	1420	U→C	Phe_{156}→Ser
			NS4B	**7196**[b]	**A→C**	**Leu_{112}→Phe**
SLE/DEN4-436,437	GGTR	42	**NS4B**	**7196**[b]	**A→C**	**Leu_{112}→Phe**
SLE/DEN4-654,655	GGTR	46	NS2B	4367	A→G	Ile_{67}→Met
			NS4B	**7196**[b]	**A→C**	**Leu_{112}→Phe**

[a] Numbering indicates amino acid position within protein. Only coding changes and nucleotide substitutions within the UTRs are indicated in this table.

[b] Vero cell adaptation mutations (non-adventitious) that were incorporated into the cDNA clone are indicated in bold.

Table 2

Neuroinvasiveness and neurovirulence of SLE/DEN4 viruses in mice.

Virus	C/prM junction	Clone	Neuroinvasiveness in adult SCID mice LD_{50} (PFU)[a]	Neurovirulence in suckling SW mice LD_{50} (PFU)[b]
SLE	-	wt	3.2	0.4
SLE/DEN4	GGTR	549	$<10^4$	$>10^2$
SLE/DEN4	GGTR	551	$>10^6$	4.4
SLE/DEN4	TSGR	554	$>10^6$	$>10^3$
SLE/DEN4Δ30	GGTR	545	$>10^5$	2.2
SLE/DEN4Δ30	GGTR	546	$>10^5$	3.0
SLE/DEN4Δ30	TSGR	609	$>10^5$	2.9
SLE/DEN4-436,437	GGTR	41	$>10^4$	$>10^3$
SLE/DEN4-654,655	GGTR	46	$>10^5$	428

[a] 21 day-old SCID mice were inoculated intraperitoneally with serial 10-fold dilutions of indicated virus and then monitored for moribundity for 49 days.

[b] 3 day-old Swiss Webster mice were inoculated intracerebrally with serial 10-fold dilutions of indicated virus and then monitored for moribundity for 21 days.

Table 3

Replication and immunogenicity of parental SLE and chimeric viruses in rhesus monkeys

Virus[a]	Clone	No. of monkeys	% with viremia	Mean no. of days with viremia	Mean peak virus titer ± SE (log_{10}PFU/ml)[b]	Geometric mean serum neutralizing antibody titer[c]	Seroconversion[d] (%)
SLE	wt	10	90	3.5	2.1 ± 0.2	39	90
SLE/DEN4	551	6	83	2.2	1.1 ± 0.2	109	100
SLE/DEN4Δ30	545	4	0	0	< 0.7	< 5	0
SLE/DEN4-436,437	41	4	75	2.0	1.0 ± 0.2	28	75
SLE/DEN4-654,655	46	4	0	0	< 0.7	11	0

[a] Groups of rhesus monkeys were inoculated SC with 10^5 PFU of indicated virus. Serum was collected on day 0-6, 8, and 10 for viremia assay and day 28 for antibody titer determination.

[b] Virus titer in serum was determined by plaque assay in Vero cells. Mean peak virus titer of SLE-infected monkeys was significantly different from that of other groups as determined by Tukey-Kramer post hoc test ($P<0.05$).

[c] Plaque reduction (60%) neutralizing antibody titers were determined using SLE/DEN4 as target virus. The reciprocal dilution is reported.

[d] Seroconversion defined as a 4-fold or greater increase in serum neutralizing antibody level to SLE/DEN4 on day 28.