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The neurovirulence and neuroinvasiveness of chimeric tick-borne encephalitis/dengue virus can be attenuated by introducing defined mutations into the envelope and NS5 protein genes and the 3′ non-coding region of the genome

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ABSTRACT

Tick-borne encephalitis (TBE) is a severe disease affecting thousands of people throughout Eurasia. Despite the use of formalin-inactivated vaccines in endemic areas, an increasing incidence of TBE emphasizes the need for an alternative vaccine that will induce a more durable immunity against TBE virus (TBEV). The chimeric attenuated virus vaccine candidate containing the structural protein genes of TBEV on a dengue virus genetic background (TBEV/DEN4) retains a high level of neurovirulence in both mice and monkeys. Therefore, attenuating mutations were introduced into the envelope (E_{315}) and NS5 ($NS5_{654,655}$) proteins, and into the 3′ non-coding region (Δ30) of TBEV/DEN4. The variant that contained all three mutations ($v\Delta30/E_{315}/NS5_{654,655}$) was significantly attenuated for neuroinvasiveness and neurovirulence and displayed a reduced level of replication and virus-induced histopathology in the brains of mice. The high level of safety in the central nervous system indicates that $v\Delta30/E_{315}/NS5_{654,655}$ should be further evaluated as a TBEV vaccine.

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Introduction

Tick-borne encephalitis (TBE) is a severe neurological disease caused by several antigenically related RNA viruses within the tick-borne encephalitis virus (TBEV) serocomplex of the *Flaviviridae* family (Lindenbach et al., 2007). Viruses in the TBEV complex are endemic to Europe, Asia, and North America and include the European, Siberian, and Far Eastern subtypes of TBEV as well as Kyasanur forest disease, Langat (LGT), louping ill (LI), Omsk hemorrhagic fever, and Powassan viruses. TBEV is transmitted in nature to various mammals through the bite of an infected tick. Humans serve as incidental hosts and can also become infected by ingesting unpasteurized milk products obtained from infected ruminants or by accidental exposure via aerosol (Gritsun et al., 2003).

TBEV creates a significant public health burden in endemic areas due to neurological disease in humans, leading to severe, long-term neurological complications with up to 30% mortality (Gritsun et al., 2003). Symptoms of TBE range from non-specific febrile illness to meningoencephalitis, although the majority of TBEV infections remain subclinical. However, more than 10,000 hospitalized cases of TBEV are reported annually in Europe and Russia, indicating a much higher incidence of infection than is generally recognized (Suss, 2003). Furthermore, there has been an increase in the number of TBE cases during the last 20 years, likely due to many factors including climate change, social and economic changes in land use, and low vaccination coverage rates in endemic regions (Kunze, 2006; Randolph, 2008).

The TBEV genome is a positive-sense single-stranded RNA that is approximately 11 kb in length and contains 5′ and 3′ non-coding regions (NCR) flanking a single open reading frame that encodes a polyprotein. The polyprotein is processed by viral and cellular proteases into three structural proteins (capsid (C), premembrane (prM), and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Lindenbach et al., 2007). The non-structural proteins regulate virus RNA replication and translation and attenuate host antiviral responses, whereas the structural proteins mediate virus attachment, membrane fusion, virus assembly, and elicit protective immunity in the host (Diamond, 2009; Lindenbach et al., 2007; Robertson et al., 2009).

Despite several attempts over the last 60 years to develop a safe, efficacious live attenuated virus vaccine against TBEV (Gritsun et al., 2003), it has been difficult to derive one that is satisfactorily attenuated for the central nervous system (CNS) of humans. However, four formalin-inactivated TBEV vaccines are licensed for use in

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Europe, Canada, or Russia (Baxter FSME-IMMUN® and Novartis Encepur®, derived from Central European TBEV strains Neudoerfl and K23, respectively; Institute of Poliomyelitis and Viral Encephalitides TBEV vaccine and Microgen Encevir®, derived from Far Eastern strains Sofjin and 205, respectively) (Barrett et al., 2004; Leonova and Pavlenko, 2009). Although protective levels of neutralizing antibodies in humans are induced after primary immunization with three doses of the inactivated virus vaccine, booster immunizations are required every 3 to 5 years since neutralizing antibody titers decline over time and with age (Barrett et al., 2004). Nevertheless, extensive TBEV vaccination in Austria has demonstrated that use of the inactivated TBEV vaccine in endemic areas results in a dramatic decline of TBE incidence (Barrett et al., 2004; Kunze, 2006), indicating a high level of efficacy with the inactivated virus vaccine. Although TBEV vaccination in Austria has been highly successful, several practical concerns have arisen with use of the inactivated virus vaccines, including the long schedule of primary immunization, the need for repeated booster vaccinations due to the relatively short duration of immunity, and the high cost of manufacture, all of which contribute to the relatively high cost of immunization. Furthermore, the lower immune responsiveness in the elderly and the rare occurrence of severe TBE disease due to incomplete protection of vaccinees in endemic areas are also of concern with the inactivated virus vaccines (Andersson et al., 2010; Bender et al., 2004; Brauchli et al., 2008; Kleiter et al., 2006; Plisek et al., 2008). Use of a live attenuated TBEV vaccine that induces long-lasting protective immunity is the most likely alternative approach to prevent TBE, since one or two doses of live attenuated yellow fever (YF) virus 17D vaccine (Monath, 2005) and Japanese encephalitis (JE) virus SA 14-14-2 vaccine (Halstead and Tsai, 2004) have been shown to provide immunity for at least 10 years.

In an effort to develop an efficacious live attenuated vaccine against TBE, a chimeric TBEV/DEN4 virus was previously generated by replacing the prM and E protein genes of non-neuroinvasive, mosquito-borne dengue type 4 (DEN4) virus with the corresponding genes of highly virulent Far Eastern TBEV strain Sofjin (Pletnev et al., 1992). Although TBEV/DEN4 exhibits greatly reduced neuroinvasiveness in immunocompetent mice compared to the TBEV parental virus and provides complete protection against lethal challenge with TBEV strain Sofjin, it retains neuroinvasiveness in immunodeficient mice and a substantial level of neurovirulence in suckling mice (Pletnev et al., 1992, 1993; Rumyantsev et al., 2006a). Therefore, in an effort to satisfactorily attenuate TBEV/DEN4 for neuroinvasiveness and neurovirulence, the chimera was modified by introducing a genetically stable 30 nucleotide deletion (Δ30) in the 3′ NCR of the DEN4 portion of the genome since this mutation was previously shown to attenuate DEN1 and DEN4 viruses as well as chimeric West Nile, St. Louis encephalitis, and DEN2 (WN/DEN4, SLE/DEN4, DEN2/DEN4) vaccine candidates in mice, monkeys, and/or humans (Blaney et al., 2008; Durbin et al., 2001, 2006a,b; Pletnev et al., 2006; Whitehead et al., 2003). Although introduction of the Δ30 mutation into the TBEV/DEN4 genome has a significant attenuating effect on neuroinvasiveness in immunodeficient mice and on the level of viremia in rhesus monkeys, TBEV/DEN4Δ30 still retains a high level of neurovirulence in suckling mice (Rumyantsev et al., 2006a) and a moderate level of neurovirulence in rhesus monkeys (Maximova et al., 2008). Hence, a further reduction in the level of neurovirulence of the chimeric TBEV/DEN4 or TBEV/DEN4Δ30 virus was needed before it could be considered for evaluation in humans.

In the present study, further attenuation of the neuroinvasiveness and neurovirulence of TBEV/DEN4 and TBEV/DEN4Δ30 was sought by introducing amino acid substitutions that had previously been shown to reduce flavivirus replication in mouse brain or in neuronal cell lines (Hanley et al., 2002; Rumyantsev et al., 2006b). Rumyantsev et al. (2006b) demonstrated that a single Lys → Glu substitution in the E protein at position 315 (E_{315}) in LGT virus reduces neuroinvasiveness in immunodeficient mice and decreases its replication in human and murine neuronal cells. In addition, Hanley et al. (2002) demonstrated that several paired charge-to-alanine substitutions in the NS5 polymerase protein, including an AspArg → AlaAla substitution in NS5 at positions 654 and 655 ($NS5_{654,655}$), attenuate DEN4 virus neurovirulence for suckling mice. In the current studies, the substitutions at residues E_{315} and/or $NS5_{654,655}$ were introduced into either the TBEV/DEN4 or TBEV/DEN4Δ30 genome and the phenotypes of the resulting viruses were evaluated *in vitro* and *in vivo*. The mutant viruses were attenuated for replication in cell culture and attenuated for neuroinvasiveness and neurovirulence in mice compared to TBEV/DEN4 and TBEV/DEN4Δ30 viruses.

Results

Derivation of chimeric TBEV/DEN4 and TBEV/DEN4Δ30 mutant viruses

Eight chimeric viruses were used in these studies. Two viruses, TBEV/DEN4 and TBEV/DEN4Δ30, have been described previously (Pletnev et al., 1992; Rumyantsev et al., 2006a). Chimeric TBEV/DEN4 virus contains the structural prM and E protein genes of the Far Eastern TBEV strain Sofjin as well as the remaining capsid and non-structural protein genes of DEN4 virus, whereas TBEV/DEN4Δ30 also contains the Δ30 deletion within the 3′ NCR of the DEN4 portion of the genome. Since both TBEV/DEN4 and TBEV/DEN4Δ30 viruses retain substantial neurovirulence in suckling mice (Rumyantsev et al., 2006a), two sets of amino acid substitutions within the E (Lys_{315} → Asp) and NS5 ($AspArg_{654,655}$ → AlaAla) protein genes were introduced, singly or in combination, into either the TBEV/DEN4 or TBEV/DEN4Δ30 infectious cDNA clone in order to further attenuate the viruses. Three new chimeric TBEV/DEN4 virus mutants (designated as vE_{315}, $vNS5_{654,655}$, and $vE_{315}/NS5_{654,655}$) and three new TBEV/DEN4Δ30 virus mutants (designated as $v\Delta30/E_{315}$, $v\Delta30/NS5_{654,655}$, and $v\Delta30/E_{315}/NS5_{654,655}$) containing these substitutions were recovered from Vero cells and biologically cloned by terminal dilution. Sequence analysis encompassing the regions of these mutations revealed that all recovered viruses contained the desired mutations. Mutant viruses replicated to titers of at least $10^{6.4}$ PFU/ml when amplified in Vero cells at 32 °C, a temperature that was considered permissive for viral replication based on previous studies with LGT and DEN4 viruses containing the E_{315} and $NS5_{654,655}$ amino acid substitutions, respectively (Hanley et al., 2002; Rumyantsev et al., 2006b).

In vitro characterization of chimeric viruses

The temperature sensitivity (*ts*) phenotypes of chimeric TBEV/DEN4 and TBEV/DEN4Δ30 mutant viruses were determined by titration of each virus in simian Vero cells and in human neuronal SH-SY5Y or LN-18 cells at various temperatures since previous studies with LGT and DEN4 viruses have shown that a *ts* phenotype in cell culture is often associated with attenuation *in vivo* (Hanley et al., 2002; Rumyantsev et al., 2006a,b). TBEV/DEN4, TBEV/DEN4Δ30, and vE_{315} viruses were not *ts* in any of the cell lines examined (Table 1). However, mutating the $NS5_{654,655}$ residues, which are *ts* in the parental DEN4 mutant virus (Hanley et al., 2002), resulted in a *ts* phenotype for the TBEV/DEN4Δ30 virus. Although the E_{315} and Δ30 mutations did not confer *ts* phenotypes themselves, combining them with the $NS5_{654,655}$ mutation greatly increased the level of temperature sensitivity (Table 1). Such *ts* mutant viruses would be expected to exhibit an attenuation phenotype in mice. For example, the triple mutant virus, $v\Delta30/E_{315}/NS5_{654,655}$, was highly restricted in its replication at 37 °C and 39 °C in each cell line, suggesting that it should be restricted for replication in the brains of mice, which have a core body temperature of 37 °C.

Since small plaque (*sp*) phenotype in cell culture has also been associated with attenuation *in vivo* (Rumyantsev et al., 2006a,b; Wright et al., 2008), this characteristic was separately evaluated in Vero cells. Although none of the three individual mutations affected

Table 1
Efficiency of plaque formation of TBEV/DEN4- and TBEV/DEN4Δ30-derived viruses in Vero, SH-SY5Y, and LN-18 cell lines at permissive and restrictive temperatures.

Virus	Virus titer ($\log_{10}$ PFU/ml) at the indicated temperature (°C)																		
	Vero							SH-SY5Y						LN-18					
	32	35	37	39	Δ[a]	*ts*[b]	*sp*[c]	32	35	37	39	Δ	*ts*	32	35	37	39	Δ	*ts*
TBEV/DEN4	6.4	6.4	6.1	6.0	0.4	−	−	6.4	6.4	6.1	6.1	0.3	−	5.7	5.5	5.6	5.3	0.4	−
vE_{315}	6.8	6.7	6.4	6.0	0.8	−	−	7.3	7.1	6.5	6.0	1.3	−	6.0	6.3	5.8	4.4	1.6	−
$vNS5_{654,655}$	6.9	6.9	6.8	5.2	1.7	−	−	7.0	6.7	6.9	6.0	1.0	−	6.0	6.1	6.0	<**1.7**	>**4.3**	+
$vE_{315}/NS5_{654,655}$	6.5	5.6	**4.0**	<**1.7**	>**4.8**	+	+	7.0	6.3	6.0	<**1.7**	>**5.3**	+	5.3	4.3	**2.0**	<**1.7**	>**3.6**	+
TBEV/DEN4Δ30	7.8	7.5	7.1	6.0	1.8	−	−	7.8	7.9	7.3	6.5	1.3	−	6.9	6.7	6.3	5.0	1.9	−
$v\Delta30/E_{315}$	6.7	6.7	6.3	5.7	1.0	−	+	6.8	6.5	6.4	5.8	1.0	−	4.8	5.3	4.9	3.0	1.8	−
$v\Delta30/NS5_{654,655}$	7.4	6.9	**4.0**	**2.8**	**4.6**	+	+	7.2	6.7	6.7	**2.6**	**4.6**	+	6.5	6.1	**4.0**	**1.3**	**5.2**	+
$v\Delta30/E_{315}/NS5_{654,655}$	6.6	5.8	**3.0**	<**1.0**	>**5.6**	+	+	6.4	6.0	**3.7**	<**1.0**	>**5.4**	+	4.0	3.0	**2.0**	<**1.0**	>**3.0**	−

[a] Reduction in virus titer at 39 °C compared to the virus titer at 32 °C.
[b] A mutant virus was defined as having a *ts* phenotype if its shutoff temperature was ≤39 °C. The shutoff temperature for plaque formation was defined as the lowest temperature at which the reduction in virus titer at a restrictive temperature compared to its titer at 32 °C was 100-fold greater than the reduction in parental virus titer between the same two temperatures (formula for calculating *ts* phenotype: $\geq 2.0 \log_{10}$ PFU/ml = $(\text{Titer}_{32\,°C} - \text{Titer}_{39\,°C})_{\text{Chimeric virus}} - (\text{Titer}_{32\,°C} - \text{Titer}_{39\,°C})_{\text{Parental virus}}$). Values in bold indicate $\geq 2.0 \log_{10}$ PFU/ml reduction in titer at the indicated temperature.
[c] The *sp* phenotype was defined as virus mean plaque diameter ≤50% of TBEV/DEN4 or TBEV/DEN4Δ30 virus on Vero cells at 32 °C. Mean plaque diameter of TBEV/DEN4 and TBEV/DEN4Δ30 following a 6-day incubation at 32 °C was 1 mm.

plaque size at 32 °C in Vero cells, each recombinant virus bearing two or more of these mutations acquired the *sp* phenotype (Table 1). Thus, $v\Delta30/E_{315}/NS5_{654,655}$ was highly attenuated *in vitro* as shown by *ts* and *sp* phenotypes, indicating that it may be attenuated *in vivo*.

Neurovirulence of chimeric viruses in suckling mice

The neurovirulence of TBEV/DEN4 and TBEV/DEN4Δ30 mutant viruses was evaluated in 3-day-old mice by estimating LD_{50} values after intracerebral (IC) inoculation. Mice of this age were used because they are a highly sensitive model for evaluation of flavivirus neurovirulence. Consistent with previous observations (Rumyantsev et al., 2006a), introduction of the Δ30 deletion into the TBEV/DEN4 genome did not alter the virus LD_{50} or the average survival time (AST) after IC inoculation (Table 2). However, introduction of the E_{315} or $NS5_{654,655}$ mutation into TBEV/DEN4 increased the LD_{50} of the resulting mutant viruses by 8- to 20-fold, respectively, while co-introduction of both substitutions significantly increased the LD_{50} by 51-fold ($p<0.05$) (Table 2). Furthermore, the AST of these mice increased from 7.4 days to 8.6, 12.2, and >21 days for viruses containing the E_{315}, $NS5_{654,655}$, and $E_{315}/NS5_{654,655}$ mutations, respectively. These findings indicate that the E_{315} and $NS5_{654,655}$ mutations, individually or in combination, decreased the neurovirulence of TBEV/DEN4 for 3-day-old mice.

Table 2
Neurovirulence of TBEV/DEN4- and TBEV/DEN4Δ30-derived viruses in 3-day-old Swiss Webster mice.

Virus	LD_{50} (PFU)[a]	LD_{50} fold reduction[b]	AST (days)[c]
TBEV/DEN4	0.8	–	7.4
vE_{315}	6.6	8	8.6
$vNS5_{654,655}$	16.2	20	12.2
$vE_{315}/NS5_{654,655}$	40.8*	51	>21
TBEV/DEN4Δ30	1.0	–	7.0
$v\Delta30/E_{315}$	4.1	4	11.9
$v\Delta30/NS5_{654,655}$	40.7*	41	16.6
$v\Delta30/E_{315}/NS5_{654,655}$	487*	487	>21

* Statistically significant differences ($p<0.05$, nominal logistic fit for survival) between LD_{50} values of parental (TBEV/DEN4 and TBEV/DEN4Δ30) and mutant viruses.
[a] Multiple litters of approximately 10 mice (range 9–16) were inoculated IC with 10-fold serial dilutions of virus ranging from 0.1 to 10 PFU of TBEV/DEN4 or TBEV/DEN4Δ30 and from 1 to 10^4 PFU for mutant viruses to determine their LD_{50} values. Mice were monitored for signs of encephalitis for 21 days and moribund mice were euthanized.
[b] Fold-reduction of neurovirulence of mutant virus compared to TBEV/DEN4 or TBEV/DEN4Δ30 parental viruses.
[c] Average survival times (AST) of mice following IC inoculation with 10 PFU of virus.

A similar effect on neurovirulence was observed when the E_{315} and $NS5_{654,655}$ mutations were introduced into the TBEV/DEN4Δ30 parental virus. Although moderate decreases in neurovirulence were noted for TBEV/DEN4-derived viruses, more substantial decreases in neurovirulence were observed for TBEV/DEN4Δ30 mutant viruses containing either individual substitutions at E_{315} or $NS5_{654,655}$, or both substitutions ($E_{315}/NS5_{654,655}$), as demonstrated by 4- to 487-fold increases in LD_{50} values (Table 2). In addition, an increase of the AST (11.9, 16.6, or >21 days) was observed with TBEV/DEN4Δ30 viruses containing E_{315}, $NS5_{654,655}$, or $E_{315}/NS5_{654,655}$ mutations, respectively. Introduction of $NS5_{654,655}$ or $E_{315}/NS5_{654,655}$ into TBEV/DEN4Δ30 attenuated the virus to a greater extent than E_{315}, $NS5_{654,655}$, or Δ30 individually. Additionally, introducing $NS5_{654,655}$ or both E_{315} and $NS5_{654,655}$ substitutions significantly attenuated TBEV/DEN4Δ30 ($p<0.05$) (Table 2). Although the Δ30 mutation did not seem to alter neurovirulence on its own, its introduction together with E_{315} and/or $NS5_{654,655}$ into TBEV/DEN4 had an additive attenuating effect, as shown by greater increases in LD_{50} and AST compared to those observed for viruses that contained the individual mutations.

Genomic sequence of the TBEV/DEN4Δ30 mutant viruses

Since TBEV/DEN4Δ30-derived mutant viruses were more attenuated in tissue culture and in suckling mice compared to the TBEV/DEN4-derived mutant viruses, they were suggested to be safer vaccine candidates than the TBEV/DEN4 viruses. Therefore, the TBEV/DEN4Δ30-derived viruses were selected for additional characterization. The entire consensus sequence was first determined for TBEV/DEN4, TBEV/DEN4Δ30, and all TBEV/DEN4Δ30 mutant viruses. Several adventitious mutations, which are common for recombinant DEN4-based flaviviruses recovered and passaged in Vero cell culture, occurred among the mutant viruses (Blaney et al., 2002, 2003, 2008; Pletnev et al., 2006; Rumyantsev et al., 2006a). In addition to the Δ30 deletion, the consensus sequence of the biologically cloned TBEV/DEN4Δ30 differed from that of TBEV/DEN4 by three amino acid substitutions (prM $Asp_{54} \rightarrow Tyr$, NS3 $Ile_{123} \rightarrow Thr$, and NS4B $Leu_{112} \rightarrow Ser$), as described previously (Rumyantsev et al., 2006a). However, despite these genetic differences, both viruses replicated efficiently in neuronal cells and were also highly neurovirulent for suckling mice, as shown by comparable LD_{50} values. These data indicate that the adventitious mutations had no detectable effect on the observed properties of TBEV/DEN4Δ30 virus *in vitro* and *in vivo*.

Adventitious mutations also occurred in all three TBEV/DEN4Δ30 mutant viruses (Table 3). Four adventitious mutations occurred in $v\Delta30/E_{315}$ virus, including three that encoded amino acid substitutions (prM $Asp_{54} \rightarrow Tyr$, NS2A $Ile_{198} \rightarrow Leu$, and NS4B $Thr_{105} \rightarrow Ile$).

Table 3
Mutations identified in TBEV/DEN4Δ30 and its derivative viruses after recovery and passage in Vero cells.

Virus	Nucleotide position[a]	Nucleotide change[b]	Gene	Amino acid change and position[b,c]
TBEV/DEN4Δ30	618	G→U	prM	Asp_{54}→Tyr
	4724	U→C	NS3	silent
	4906	U→C	NS3	Ile_{123}→Thr
	7177	U→C	NS4B	Leu_{112}→Ser
vΔ30/E_{315}	618	G→U	prM	Asp_{54}→Tyr
	1893	**A→G**	**E**	**Lys_{315}→Asp**
	1895	**A→C**		
	4086	A→C	NS2A	Ile_{198}→Leu
	7156	C→U	NS4B	Thr_{105}→Ile
	7626	C→U	NS5	silent
vΔ30/$NS5_{654,655}$	1953	A→G	E	Thr_{335}→Ala
	4821	G→A	NS3	Val_{95}→Ile
	6959	A→G	NS4B	silent
	7352	A→G	NS4B	silent
	7610	G→A	NS5	silent
	9538	**A→C**	**NS5**	**Asp_{654}→Ala**
	9539	**C→A**		
	9540	**A→G**	**NS5**	**Arg_{655}→Ala**
	9541	**G→C**		
vΔ30/E_{315}/$NS5_{654,655}$	1668	C→U	E	Arg_{240}→Trp
	1893	**A→G**	**E**	**Lys_{315}→Asp**
	1895	**A→C**		
	7178	A→U	NS4B	Leu_{112}→Phe
	9538	**A→C**	**NS5**	**Asp_{654}→Ala**
	9539	**C→A**		
	9540	**A→G**	**NS5**	**Arg_{655}→Ala**
	9541	**G→C**		

[a] Numbering of nucleotide positions reflects relative nucleotide positions within the TBEV/DEN4Δ30 chimeric virus (GenBank accession no. FJ828987).
[b] Nucleotide and amino acid sequence changes that occurred during the recovery of virus from the cDNA clone. Bold changes are mutated positions that have been introduced by site-directed mutagenesis during these studies.
[c] Amino acid position is calculated from the first codon of each individual protein coding region.

Two of these substitutions (prM_{54} and $NS4B_{105}$) have been previously identified in other viruses as Vero cell adaptation mutations (Blaney et al., 2001, 2002, 2003, 2008; Rumyantsev et al., 2006a). Five adventitious mutations were identified throughout the consensus sequence of vΔ30/$NS5_{654,655}$ virus, two of which encoded amino acid substitutions (E Thr_{335}→Ala and NS3 Val_{95}→Ile). The triple mutant virus, vΔ30/E_{315}/$NS5_{654,655}$, contained an additional two amino acid substitutions (E Arg_{240}→Trp and NS4B L_{112}→Phe) compared to TBEV/DEN4Δ30 virus, of which $NS4B_{112}$ has been previously identified as a Vero cell adaptation mutation (Blaney et al., 2001, 2002, 2003, 2008; Rumyantsev et al., 2006a). Although adventitious mutations occurred in all TBEV/DEN4Δ30-derived viruses, they did not appear to affect the virus phenotype as the introduced mutations demonstrated the same pattern of attenuation, regardless of whether they were introduced in the TBEV/DEN4 or TBEV/DEN4Δ30 backbone (i.e., E_{315}>$NS5_{654,655}$>E_{315}/$NS5_{654,655}$).

Replication of TBEV/DEN4Δ30 mutant viruses in suckling mouse brain

Since TBEV/DEN4Δ30-based mutant viruses were more attenuated for neurovirulence in mice compared to the TBEV/DEN4 derivatives, the replication kinetics and genetic stability of the viruses were evaluated in the brains of suckling mice (Fig. 1). Following IC inoculation with 10^3 PFU of virus, TBEV/DEN4Δ30 rapidly reached high titers ($10^{7.6}$ PFU/g) in mice by day 5 and caused paralysis or death on day 7. However, the mutant viruses replicated to lower titers and achieved peak virus titers later than TBEV/DEN4Δ30. vΔ30/E_{315} and vΔ30/$NS5_{654,655}$ demonstrated 25- to 32-fold decreases in their peak titers compared to TBEV/DEN4Δ30. vΔ30/E_{315}/$NS5_{654,655}$ was the most attenuated in suckling mouse brains, as its peak titer was approximately 20,000-fold lower than TBEV/DEN4Δ30. Furthermore,

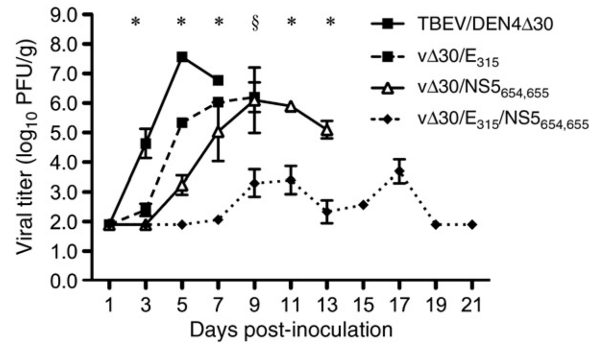

Fig. 1. Virus replication kinetics of TBEV/DEN4Δ30 or its derived mutants in the brains of suckling mice. Litters of 5-day-old Swiss mice were inoculated IC with 10^3 PFU of virus. Brains of at least three mice per group were harvested on odd days post-inoculation and quantitated for virus titers by immunofocus assay on Vero cells; the mean virus replication titers ± SE are shown. Asterisks indicate that the vΔ30/E_{315}/$NS5_{654,655}$ mutant replicates significantly lower than the remaining viruses on the indicated day (unpaired *t* test or one-way ANOVA followed by Tukey post hoc test, $p<0.05$). The § indicates that only two mouse brains were harvested in the vΔ30/E_{315} group on day 9, as they were the only remaining animals in the group. The limit of detection was 2.0 log_{10} PFU/g. Lines end at the day when all animals succumbed to infection, with the exception of day 21 when the experiment was terminated.

the triple mutant virus replicated significantly lower in the brains of suckling mice than all viruses compared on days 3, 5, 7, 11, and 13 ($p<0.05$).

To investigate genetic stability of the introduced mutations in the TBEV/DEN4Δ30-derived mutant viruses, genomic sequence of the virus RNA present in the brains of mice was obtained for all viruses at the endpoint of study (paralysis or on the last day with detectable virus) by directly sequencing RT-PCR cDNA fragments without passage of the virus in cell culture (Table 4). Sequence analysis of vΔ30/E_{315} from 12 infected mice on days 8 and 9 and vΔ30/$NS5_{654,655}$ from seven infected mice on day 13 demonstrated that the engineered mutations at E_{315}, $NS5_{654,655}$, and Δ30 were stable (Table 4). Furthermore, all virus genomes from six brains harvested on days 15 and 17 from mice inoculated with the triple mutant virus (vΔ30/E_{315}/$NS5_{654,655}$) were found to contain all three sets of introduced mutations. Only one mouse was paralyzed in the triple mutant group (day 17); however, all introduced mutations were stable in the virus present in the brain of this animal.

Neuroinvasiveness of chimeric viruses in immunodeficient mice

Since parental TBEV/DEN4 lost its ability to spread from a peripheral site of inoculation to the CNS in immunocompetent mice (Pletnev et al., 1993), we investigated the neuroinvasive properties of our modified TBEV/DEN4Δ30 viruses in immunodeficient mice.

Table 4
Genetic stability of introduced mutations in TBEV/DEN4Δ30 mutant viruses present in the brains of 5-day-old mice.

Virus[a]	Day of isolation[b]	No. tested	No. mutations changed/No. tested[c]		
			Δ30	E_{315}	$NS5_{654,655}$
vΔ30/E_{315}	8	10	0/10	0/10	–
	9	2	0/2	0/2	–
vΔ30/$NS5_{654,655}$	13	7	0/7	–	0/7
vΔ30/E_{315}/$NS5_{654,655}$	15	2	0/2	0/2	0/2
	17	4	0/4	0/4	0/4

[a] Five-day-old mice were inoculated IC with 10^3 PFU of indicated virus.
[b] Brains of mice were harvested on indicated day, and virus RNA was isolated from brain homogenate to determine virus genomic sequence.
[c] The virus genome regions encompassing the introduced Δ30, E_{315}, or $NS5_{654,655}$ mutations were directly sequenced from brain homogenates to determine stability of the mutations. Dashed lines indicate that no mutation was originally introduced at this position.

Table 5
Neuroinvasiveness of TBEV/DEN4, TBEV/DEN4Δ30, and TBEV/DEN4Δ30 mutant derivatives in immunodeficient SCID mice.

Virus	Dose (PFU)	AST (day)	No. moribund/No. tested (%)
TBEV/DEN4	10^5	23	6/10 (60)
TBEV/DEN4Δ30	10^5	22	10/56 (18)*
vΔ30/E_{315}	10^5	32	3/33 (9)*
vΔ30/$NS5_{654,655}$	10^5	>49	0/33 (0)*,§
vΔ30/E_{315}/$NS5_{654,655}$	10^5	>49	0/41 (0)*,§

* All TBEV/DEN4Δ30 derivatives were significantly different from TBEV/DEN4 ($p<0.05$, Kaplan–Meier survival curve followed by Tukey post hoc test).
§ vΔ30/$NS5_{654,655}$ and vΔ30/E_{315}/$NS5_{654,655}$ were significantly different from TBEV/DEN4Δ30 ($p<0.05$, Kaplan–Meier survival followed by Tukey post hoc test).

Groups of SCID mice were inoculated intraperitoneally (IP) with 10^5 PFU of chimeric virus and were assessed for AST, morbidity, and virus replication in the brain. Although introduction of the Δ30 deletion into TBEV/DEN4 has no effect on neurovirulence, a decrease in neuroinvasiveness was observed for TBEV/DEN4Δ30 virus, as shown by a significant reduction in morbidity (from 60% to 18%) of mice compared to that of TBEV/DEN4 ($p<0.05$) (Table 5). Since these findings are consistent with our previous observations (Rumyantsev et al., 2006a), we investigated the ability of Δ30 in combination with E_{315} and/or $NS5_{654,655}$ substitutions to further reduce neuroinvasiveness. Although the combination of E_{315} with Δ30 increased the AST and decreased the morbidity compared to TBEV/DEN4Δ30, the difference was not significant. However, introduction of $NS5_{654,655}$ or the combination of E_{315}/$NS5_{654,655}$ mutations into the TBEV/DEN4Δ30 genome greatly diminished ($p<0.05$) the ability of the chimeric virus to invade the CNS from a peripheral site of inoculation and cause neurological disease (Table 5).

We next investigated the ability of the TBEV/DEN4Δ30 mutant viruses to invade and replicate in the CNS after peripheral inoculation. Adult immunodeficient SCID mice were inoculated IP with 10^5 PFU of virus, and brains were harvested on odd days post-infection. TBEV/DEN4 replicated in the brains between $10^{6.5}$ and $10^{8.5}$ PFU/g on days 7 to 21 and was significantly different from all viruses on all days ($p<0.05$), except for TBEV/DEN4Δ30 on days 17 and 21 (Fig. 2). Although TBEV/DEN4Δ30 virus replication was not detected in peripheral tissues, such as the spleen and liver (data not shown), virus was detected in the brain on days 11 to 21 ($10^{2.1}$ to $10^{6.6}$ PFU/g), and the mean peak virus titer ($10^{6.6}$ PFU/g) was observed on day 15 (Fig. 2). Since TBEV/DEN4Δ30 virus demonstrated the highest titers between days 13 and 19 pi, these days were chosen to harvest brains from SCID mice infected with the TBEV/DEN4Δ30 mutant derivatives in order to assess the ability of these viruses to invade and replicate in the CNS from a peripheral site of inoculation. Replication of the mutant viruses was significantly impaired ($p<0.05$), as infectious virus was not recovered from the brains of SCID mice on any of these days following IP inoculation with vΔ30/E_{315}, vΔ30/$NS5_{654,655}$, or vΔ30/E_{315}/$NS5_{654,655}$ (Fig. 2). These studies confirm the highly reduced neuroinvasive phenotype of the mutant viruses and clearly demonstrate that the introduction of both E_{315} and $NS5_{654,655}$ mutations into the TBEV/DEN4Δ30 genome attenuates the chimeric virus in mice for both neuroinvasiveness and neurovirulence to a greater extent than either mutation alone.

Neuropathology induced by chimeric viruses in mice

Since vΔ30/E_{315}/$NS5_{654,655}$ demonstrated the greatest attenuation in both suckling and immunodeficient mice, we were interested in further investigating the safety of the triple mutant virus for the CNS; therefore, the ability of this virus to induce neuropathology in mice was assessed. Brain histopathology was analyzed in groups of three adult mice inoculated IC with 10^4 PFU of TBEV/DEN4, TBEV/DEN4Δ30, or vΔ30/E_{315}/$NS5_{654,655}$ viruses on day 6, the time at which mice inoculated with TBEV/DEN4 virus succumbed to infection. At this time point, a high level of virus replication (mean virus titer $10^{4.8}$ PFU/g) was detected in the brains of TBEV/DEN4-infected mice, whereas virus titers were 32-fold lower (mean virus titer $10^{3.3}$ PFU/g) in mice inoculated with TBEV/DEN4Δ30. The mean virus titer ($10^{1.7}$ PFU/g) observed in the brains of mice infected with vΔ30/E_{315}/$NS5_{654,655}$ virus was approximately 40- to 1259-fold lower than that observed with TBEV/DEN4Δ30 and TBEV/DEN4, respectively.

Brain histopathology correlated well with the level of virus replication, as the most severe and widespread histopathology (including perivascular and parenchymal mononuclear inflammatory cell infiltration, microglial proliferation, and neuronal degeneration) was observed in mice infected with the parental TBEV/DEN4 virus (Figs. 3A, D, and G). TBEV/DEN4Δ30 induced less severe neuroinflammation (Figs. 3B, E, and H) compared to the parental TBEV/DEN4 virus, whereas virus-associated inflammatory changes were not observed in the brains of mice inoculated with vΔ30/E_{315}/$NS5_{654,655}$ (Figs. 3C, F, and I) or mock-inoculated controls (data not shown). Taken together, vΔ30/E_{315}/$NS5_{654,655}$ is highly attenuated for virus-induced neuropathology compared to the parental TBEV/DEN4 and TBEV/DEN4Δ30 viruses.

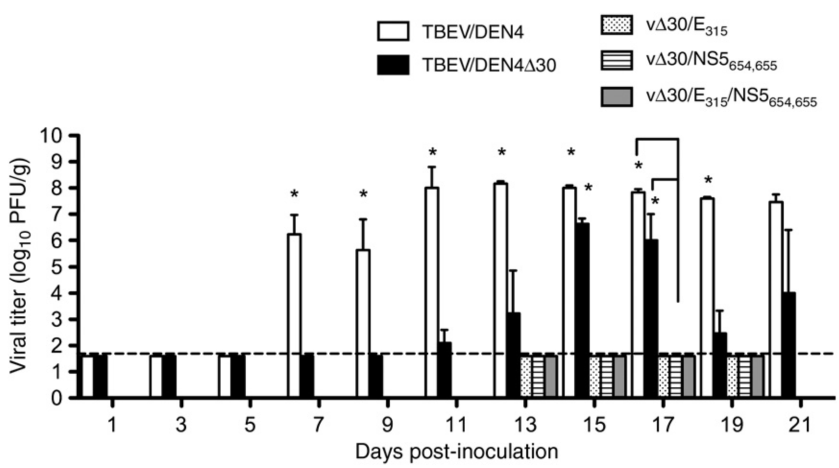

Fig. 2. Replication of TBEV/DEN4, TBEV/DEN4Δ30, and TBEV/DEN4Δ30 mutant derivatives in the brains of 3-week-old SCID mice following IP inoculation with 10^5 PFU of virus. On the indicated days, three mouse brains per group were harvested, and the virus titer of each mouse brain homogenate was determined by immunofocus assay on Vero cells. Mean virus titers are indicated ± SE. Asterisks indicate that virus replication on indicated day is significantly different from remaining viruses (unpaired *t* test or one-way ANOVA followed by Tukey post hoc test, $p<0.05$). Horizontal dashed line indicates limit of detection (1.7 log_{10} PFU/g) of the assay.

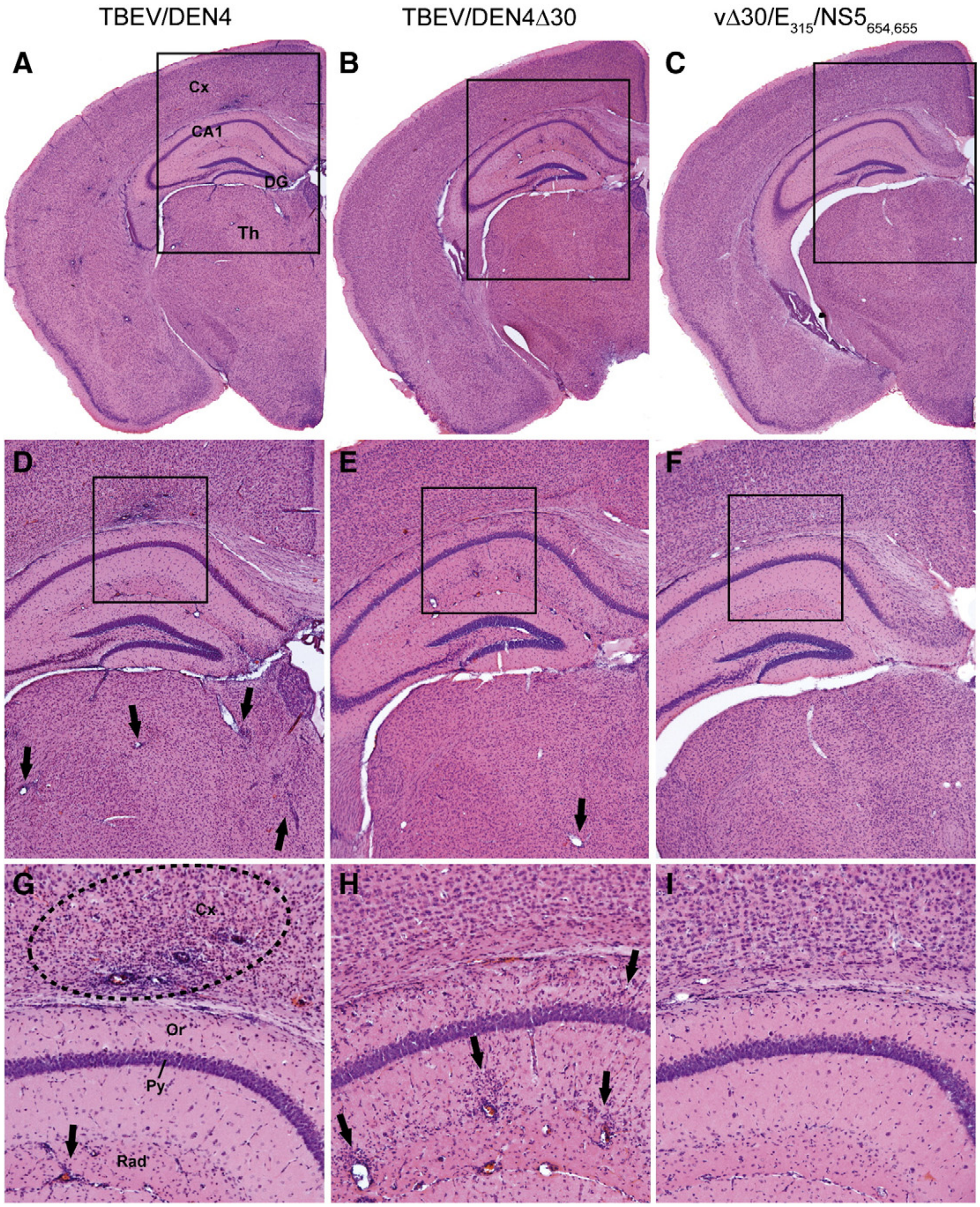

Fig. 3. Neuroinflammation in the brains of mice infected with TBEV/DEN4 (A, D, and G), TBEV/DEN4Δ30 (B, E, and H), or vΔ30/E_{315}/$NS5_{654,655}$ (C, F, and I). Representative images of neuroinflammation in the brain on day 6 from mice IC inoculated with each virus are shown (from 25 sections per brain of each of three mice; H&E staining). The boxed areas in A–C (×20) are shown in D–F at higher magnification (×40); G–I show the boxed areas in D–F at higher magnification (×100). Inflammatory foci are shown by arrows (D, E, G, and H) and the dashed circle (G). *Abbreviations:* Cx—cortex; CA1—hippocampus; DG—dentate gyrus; Th—thalamus; Or—oriens layer of the hippocampus; Py—pyramidal layer of the hippocampus; Rad—radiatum layer of the hippocampus.

Discussion

Since the development of efficacious, live attenuated vaccines requires a fine balance between virus attenuation and immunogenicity, obtaining a live attenuated virus vaccine against TBEV has been difficult. Previous live attenuated TBEV vaccine candidates have either retained a substantial level of neurovirulence (e.g., LGT, TBEV/DEN4, or TBEV/DEN4Δ30 viruses) or were weakly immunogenic against wild-type TBEV (e.g., LGT/DEN4) (Gritsun et al., 2003; Maximova et al., 2008; Wright et al., 2008). TBEV/DEN4 virus exhibits a marked reduction of neuroinvasiveness in immunocompetent adult mice (Pletnev et al., 1992, 1993); however, despite replacing ~80% of the TBEV genome with the non-structural genes of non-neurotropic mosquito-borne DEN4 virus, TBEV/DEN4 nevertheless maintains a high level of neurovirulence in suckling mice, comparable to that of wild-type TBEV strain Sofjin (Rumyantsev et al., 2006a). The level of neurovirulence observed with TBEV/DEN4 was in marked contrast to that of chimeric vaccines previously developed against TBEV (LGT/DEN4) and WN virus (WN/DEN4), which are highly attenuated for both neuroinvasiveness and neurovirulence (Pletnev and Men, 1998; Pletnev et al., 2006). Furthermore, introduction of the Δ30 deletion into TBEV/DEN4 does not result in a satisfactory level of attenuation for suckling mice or rhesus monkeys (Maximova et al., 2008; Rumyantsev et al., 2006a), despite its ability to attenuate SLE/DEN4, WN/DEN4, DEN1, DEN2/DEN4, or DEN4 viruses for mice, monkeys, and/or humans, leading to the generation of excellent vaccine candidates for the latter three viruses (Blaney et al., 2008; Durbin et al., 2001, 2006a,b; Pletnev et al., 2006; Whitehead et al., 2003). Although TBEV/DEN4Δ30 is less neuroinvasive in immunodeficient mice than TBEV/DEN4, it retains a high level of neurovirulence in both suckling mice and non-human primates and was therefore rejected as a candidate for evaluation in humans (Maximova et al., 2008; Rumyantsev et al., 2006a).

In this study, the neurovirulence of TBEV/DEN4 and TBEV/DEN4Δ30 was reduced by introducing attenuating amino acid substitutions that had previously been shown to restrict the replication of LGT or DEN4 virus in cell culture and in suckling mouse brain (Hanley et al., 2002; Rumyantsev et al., 2006b). The selected mutations were an amino acid substitution at position 315 in the structural E protein, a major contributor for *in vitro* and *in vivo* attenuation of the attenuated LGT virus strain E5-104 (Rumyantsev et al., 2006b), and a paired charge-to-alanine mutation at positions 654 and 655 in the DEN4 virus NS5 polymerase that is *ts* and results in a greater than 1000-fold reduction in replication of DEN4 in mouse brain (Hanley et al., 2002). Since previous flavivirus vaccine studies have demonstrated that a *ts* phenotype in cell culture and attenuation in mice are often associated with attenuation in non-human primates and humans (Halstead and Tsai, 2004; Rumyantsev et al., 2006a,b; Wright et al., 2008), neurovirulent TBEV/DEN4 and TBEV/DEN4Δ30 viruses were modified and tested to select a more attenuated TBEV vaccine candidate for further evaluation.

Introduction of the E_{315}, $NS5_{654,655}$, and $E_{315}/NS5_{654,655}$ mutations into either TBEV/DEN4 or TBEV/DEN4Δ30 resulted in a stepwise attenuation of the virus, both *in vitro* and *in vivo*. Specifically, the E_{315}, $NS5_{654,655}$, and $E_{315}/NS5_{654,655}$ substitutions demonstrated the lowest, moderate, and greatest level of attenuation, respectively. Furthermore, the level of attenuation observed by introducing the E_{315} or $NS5_{654,655}$ mutations into TBEV/DEN4Δ30 was greater than the level observed in the TBEV/DEN4 backbone, indicating that the set of three mutations (E_{315}, $NS5_{654,655}$, and Δ30) results in an additive level of attenuation. The cumulative effect of attenuation has been demonstrated for many viruses, including JE virus (Halstead and Tsai, 2004), LGT virus strains E5 and E5-104 (Pletnev and Men, 1998; Rumyantsev et al., 2006b), and YF virus strains Asibi and 17D (Barrett, 1997). Addition of all three mutations resulted in a virus that was highly attenuated *in vitro*, less neurovirulent in suckling mice (up to 487-fold reduction compared to TBEV/DEN4Δ30), poorly replicative in suckling mouse brain (between 500- and 20,000-fold reduction compared to TBEV/DEN4 and TBEV/DEN4Δ30 viruses), and non-neuroinvasive in immunodeficient mice.

Although the presence of adventitious mutations in the attenuated TBEV/DEN4Δ30 derivatives makes it difficult to assign an observed attenuation phenotype to the presence of the introduced mutation(s), we were able to examine the pattern of attenuation demonstrated by the individual or combined mutations in both TBEV/DEN4 and TBEV/DEN4Δ30. It was clear that $NS5_{654,655}$ was more attenuating than E_{315}, and the combination of $E_{315}/NS5_{654,655}$ had an even greater attenuating effect than the individual mutations. The similar pattern of attenuation specified by these mutations in two separate genetic backgrounds suggests that the engineered mutations, rather than the presence of adventitious mutations, contributed substantially to the observed attenuation of the TBEV/DEN4Δ30 mutant viruses for neurovirulence and neuroinvasiveness. Furthermore, the engineered substitutions in the TBEV/DEN4Δ30-derived viruses were genetically stable in the brains of suckling mice, whether introduced alone or in combination, as reverse mutations at the engineered sites were not detected in any of the 25 brain-derived virus genomes examined. Since two nucleotides were changed to introduce Asp_{315} in E and four nucleotides were changed to introduce $Ala_{654}Ala_{655}$ in NS5, the likelihood of genetic reversion in the host is considered to be low, underscoring the high level of genetic stability of these engineered mutations *in vivo*.

Interestingly, the E_{315} mutation (Lys → Asp) had only a moderate effect on attenuation in our studies with chimeric TBEV. E_{315} is found on the lateral surface of domain III (ED3), which resembles an immunoglobulin-like fold and is the putative flavivirus receptor-binding domain thought to affect virus tropism (Lindenbach et al., 2007). Therefore, altering this region may potentially disrupt secondary or tertiary structure of E and ED3. Although E_{315} is poorly conserved within the mosquito-borne flaviviruses, it is highly conserved within the tick-borne flaviviruses, as all tick-borne flaviviruses analyzed contained a Lys residue at this position. In addition, several substitutions that have been shown to affect virulence have been identified near the E_{315} residue in various flaviviruses, including YF, WN, JE, LGT, and LI viruses (Hurrelbrink and McMinn, 2003; Mandl, 2005). Many of these mutations have resulted in viruses that exhibit altered neurovirulence and neuroinvasive properties compared to their parental viruses, suggesting that this region is important for virus virulence and attenuation. These observations, along with those by Rumyantsev et al. (2006b), led us to hypothesize that the addition of a negatively charged residue (Asp) for a positively charged residue (Lys) at E_{315} would attenuate TBEV/DEN4. When the E_{315} mutation was introduced into TBEV/DEN4, it did not restrict replication of the virus in cell culture. However, it conferred an *sp* phenotype when introduced into TBEV/DEN4Δ30. Mutating E_{315} in TBEV/DEN4Δ30 reduced mouse neurovirulence 4-fold, increased AST by 4.9 days, and reduced replication in suckling mouse brains 25-fold. These data indicate that the E_{315} mutation reduced the ability of the virus to replicate systemically and within the CNS, confirming a role of E_{315} in virus tropism for the CNS.

$NS5_{654,655}$ was more attenuating than E_{315} *in vitro* and *in vivo*. Whereas the Δ30 or E_{315} mutation did not independently confer a *ts* phenotype *in vitro*, the $NS5_{654,655}$ mutation, alone or in combination with Δ30, was restrictive for growth in neuronal and Vero cell cultures, respectively, cell cultures at elevated temperatures; however, a greater restriction was observed with vΔ30/$NS5_{654,655}$ than with v$NS5_{654,655}$. Reduced *in vitro* replication of these viruses at higher temperatures (including 37 °C) indicates that the polymerase may be less thermostable and unable to replicate virus RNA efficiently, particularly in neuronal cells. Although the exact mechanism by which the $NS5_{654,655}$ mutation results in attenuation remains to be identified, this may explain the observed restricted replication of $NS5_{654,655}$ mutants in the brains of mice, which have a core body temperature of 37 °C. Consistent with the results *in vitro*, suckling mice demonstrated a 20- to 41-fold reduction of neurovirulence and approximately a 10-day delay in AST when inoculated with either v$NS5_{654,655}$ or vΔ30/$NS5_{654,655}$ virus, respectively, compared to the parental viruses. Furthermore, the combined Δ30 and $NS5_{654,655}$ mutations completely ablated virus neuroinvasiveness and replication in the brains of immunodeficient mice, even following peripheral inoculation with a high dose of virus (10^5 PFU). These data are consistent with previous studies in which the $NS5_{654,655}$ substitutions in DEN4 and SLE/DEN4 genomes result in viruses with a *ts* phenotype *in vitro* and reduced mouse neurovirulence (Blaney et al., 2008; Hanley et al., 2002). Thus, the lack of neuroinvasiveness and restricted replication in the brains of infected mice also suggest that viruses containing $NS5_{654,655}$ replicate poorly in both the periphery and CNS of the host.

NS5 is the most highly conserved flavivirus protein due to its methyltransferase and RNA-dependent RNA polymerase (RdRp) functions (Lindenbach et al., 2007). Although the 654 and 655 residues are found in the palm domain of the RdRp, they are not adjacent to the active site of the RdRp. However, the $NS5_{654,655}$ residues are highly conserved, as sequence alignment analysis demonstrates that most mosquito- and tick-borne flaviviruses contain a Glu residue at $NS5_{654}$ (only DEN4 virus contains an Asp residue at this position), and all flaviviruses contain an Arg residue at $NS5_{655}$. It is unclear whether the $NS5_{654,655}$ mutation attenuates the virus by impairing polymerase function at elevated temperatures, as mentioned above, or by other mechanisms. For example, the NS5 protein has been implicated in pathogenesis and modulation of the host innate immune response, particularly with type I interferon receptor signal inhibition for many flaviviruses (Diamond, 2009). Although the NS5 of DEN4 virus is unable to suppress STAT1 signal transduction (Park et al., 2007), DEN virus NS5 has been shown to reduce the level of STAT2 phosphorylation and nuclear translocation, resulting in IFN α/β antagonism (Ashour et al., 2009; Jones et al., 2005; Mazzon et al., 2009). Additional studies should be undertaken in order to determine whether the $NS5_{654,655}$, mutations exert an effect on type I IFN signaling, especially since these mutations are proximal to sequences essential for inhibition of type I IFN signaling in LGT virus NS5 (Park et al., 2007).

In summary, we have generated a live attenuated vaccine candidate against TBEV by introducing three sets of genetically stable attenuating mutations into TBEV/DEN4. Our current lead vaccine candidate is vΔ30/E_{315}/$NS5_{654,655}$, a virus that was highly attenuated for the CNS of mice, as demonstrated by a lack of neuroinvasiveness and virus-induced histopathology, as well as a significant reduction in neurovirulence. Furthermore, this triple mutant virus replicates in suckling mouse brains to a level comparable to another live attenuated flavivirus vaccine candidate, WN/DEN4Δ30, currently being tested in humans (Pletnev et al., 2006). However, despite restricted replication in the CNS, vΔ30/E_{315}/$NS5_{654,655}$ replicates efficiently in Vero cells, which would permit efficient vaccine manufacture. Taken together, these results indicate that vΔ30/E_{315}/$NS5_{654,655}$ achieved a high level of attenuation for the CNS and represents an improved live attenuated vaccine candidate. Furthermore, preliminary studies in mice suggest that the immunogenicity of vΔ30/E_{315}/$NS5_{654,655}$ is comparable to that of TBEV/DEN4Δ30, a virus that has already been tested in non-human primates and is able to protect against TBEV/DEN4 infection (Rumyantsev et al., 2006a); however, additional studies are underway to further analyze its immunogenicity and efficacy in mice and non-human primates as well as the infectivity of this vaccine candidate for tick and mosquito vectors.

Materials and methods

Cell culture

Simian Vero cells (World Health Organization seed, passages 143–149) were maintained in Opti-Pro Serum Free Medium (Invitrogen, Carlsbad, CA), supplemented with 4 mM L-glutamine (Invitrogen). Human neuroblastoma SH-SY5Y cells were maintained in 1:1 Minimal Essential and F12 media (Invitrogen), supplemented with 10% heat-inactivated FBS (BioWhittaker, Basel, Switzerland), whereas human glioblastoma LN-18 cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 5% heat-inactivated FBS, 4 mM of L-glutamine, and 1.5 g/L sodium bicarbonate (Invitrogen).

Construction of full-length cDNA clones and recovery of chimeric viruses

Chimeric TBEV/DEN4 virus contains the prM and E protein genes of Far Eastern subtype TBEV strain Sofjin and the remaining sequence derived from recombinant DEN4 virus, while chimeric TBEV/DEN4Δ30 virus contains an additional 30 nucleotide deletion (nucleotides 10478-10507) within the 3′ NCR of the genome. Construction of both viruses has been described previously (Pletnev et al., 1992; Rumyantsev et al., 2006a). The full-length infectious cDNA clones of TBEV/DEN4 and TBEV/DEN4Δ30 (GenBank accession numbers FJ828986 and FJ828987, respectively) were used in these studies to generate recombinant viruses containing amino acid substitutions Lys→Asp within the E protein at residue 315 (E_{315}) and/or AspArg→AlaAla within the NS5 protein at residues 654 and 655 ($NS5_{654,655}$). Although an attenuating positive-to-negative charged amino acid substitution (Lys_{315}→Glu; AAA→GAA codons) was originally mutated in the E protein gene of LGT virus (Rumyantsev et al., 2006b), an Asp residue (GAC codon) was introduced into TBEV/DEN4 and TBEV/DEN4Δ30 at this position. This substitution was chosen since it would require two nucleotide substitutions in the Asp codon to restore the Lys residue and would serve to reduce potential reversion to the positively charged Lys residue (AAA or AAG codons).

DNA fragments encompassing either DEN4 virus- or TBEV-specific sequences were sub-cloned into the pUC18 vector and each amino acid substitution was introduced through site-directed mutagenesis, as previously described (Hanley et al., 2002; Rumyantsev et al., 2006b). Mutagenic primers introducing Asp (codon GAC) at amino acid residue E_{315} (nucleotides 1893 and 1895) and AlaAla (codons GCA and GCG) at amino acid residues $NS5_{654,655}$ (nucleotides 9538-9541) were used to engineer these mutations in pUC-TBEV and pUC-DEN4c plasmid DNA, respectively. The pUC18-TBEV fragment contained unique *Nhe*I and *Xho*I restriction sites that corresponded to TBEV/DEN4 nucleotides 240-2361, while the pUC-DEN4c fragment contained unique *Sac*II and *Mlu*I sites that corresponded to TBEV/DEN4 nucleotides 9334-10418. Fragments containing the desired mutations were excised from pUC-TBEV or pUC-DENc by restriction digest and introduced into the corresponding sites of the full-length TBEV/DEN4 or TBEV/DEN4Δ30 infectious cDNA clones containing an SP6 promoter (Pletnev et al., 1992). RNA transcripts derived from the modified TBEV/DEN4 or TBEV/DEN4Δ30 cDNA clones were generated by transcription with SP6 polymerase (EpiCentre, Madison, WI) after linearization of the cDNA with *Asp*718 (Roche, Indianapolis, IN) and subsequently transfected into Vero cells using Lipofectamine (Invitrogen). Since the mutations at positions E_{315} and $NS5_{654,655}$ previously resulted in temperature sensitivity in either LGT or DEN4 virus, all mutant viruses were recovered and grown at 32 °C. The recovered derivatives of TBEV/DEN4 and TBEV/DEN4Δ30 were biologically cloned by two terminal dilutions and then amplified by two passages in Vero cells before experimental virus stocks were prepared. All experiments using TBEV/DEN4-derived viruses were conducted in BSL-3 containment laboratories at the National Institutes of Health, whereas all experiments using TBEV/DEN4Δ30-derived viruses were conducted in BSL-2 laboratories.

To determine the titer of the chimeric TBEV/DEN4 viruses, confluent monolayers of Vero cells in 24- or 48-well plates were infected with 10-fold serial dilutions of virus, incubated at 37 °C for one hour, and then were overlaid with Opti-MEM I containing 1% methylcellulose (Invitrogen), 2% heat-inactivated FBS, 4 mM L-glutamine, and 0.05 mg/ml of gentamycin. After incubation for 6 days at 32 °C, the cells were fixed for 20 min with 100% methanol, and plaques were visualized by immunostaining with TBEV-specific hyperimmune mouse ascitic fluid (ATCC) and peroxidase-labeled polymer conjugated to anti-mouse immunoglobulin (Dako Co., Carpinteria, CA).

Recombinant plasmid DNA and cDNA genomes of the recovered viruses were sequenced around the site of mutagenesis or in their entirety to verify the presence of the introduced mutations within the genome. Virus RNA was extracted from virus suspension using the QiaAmp Viral RNA mini kit (Qiagen, Valencia, CA); one-step RT-PCR was performed on the virus RNA using the Superscript One-Step kit (Invitrogen) and DEN4 virus- or TBEV-specific primers. The nucleotide consensus sequences of the virus genomes were determined through direct sequence analysis of the PCR fragments on a 3730 Genetic Analyzer using TBEV- or DEN4 virus-specific primers in BigDye terminator cycle sequencing reactions (Applied Biosystems, Foster City, CA) and were analyzed using Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, MI).

In vitro characterization of mutant viruses

Parental and mutant viruses were evaluated in a comparative study for temperature sensitivity (*ts*) and small plaque (*sp*) phenotypes by assessing virus titers at 32°, 35°, 37°, and 39 °C in simian kidney Vero, human neuroblastoma SH-SY5Y, or human glioblastoma LN-18 cells. The efficiency of plaque (EOP) formation was determined by infecting confluent monolayers of Vero, LN-18, or SH-SY5Y cells with 10-fold serially diluted virus for 1 h at 37 °C, after which Opti-MEM I overlay containing methylcellulose, FBS, and gentamycin was added. Cells were incubated for 6 days at the assigned temperature and plaques were visualized by immunostaining, as described above. A mutant was defined as having a *ts* phenotype if its shutoff temperature was ≤39 °C. The shutoff temperature for plaque formation was defined as the lowest temperature at which the reduction in virus titer at a restrictive temperature compared to its titer at 32 °C was 100-fold greater than the reduction in parental virus titer between the same two temperatures. Mutant viruses with mean plaque diameters that were ≤50% of the size of the parental TBEV/DEN4 or TBEV/DEN4Δ30 virus on Vero cells were designated as having an *sp* phenotype.

Evaluation of mutant viruses in mice

Mice were handled according to Federal and NIAID Animal Care and Use Committee regulations. To determine the neurovirulence of all chimeric TBEV/DEN4 and TBEV/DEN4Δ30 viruses, 3-day-old Swiss Webster mice (Taconic Farms, Germantown, NY), in litters of approximately 10 animals, were inoculated with 10-fold serial dilutions of virus via the intracerebral (IC) route. Three litters of mice per virus were inoculated with 0.1, 1, or 10 PFU of TBEV/DEN4 or TBEV/DEN4Δ30, whereas four litters of mice were inoculated with a dose ranging from 1 to 10^4 PFU of each mutant virus to determine their LD_{50} values. Mice were monitored for morbidity and mortality up to 21 days post-inoculation and the 50% lethal dose (LD_{50}) was determined by the Reed and Muench (1938) method. Moribund (paralyzed) mice were humanely euthanized and scored as a lethality. Significant differences between LD_{50} levels were determined by using the nominal logistic fit for survival ($p<0.05$) (JMP V8.0 software, Cary, NC).

Further studies were undertaken in litters of 5-day-old suckling Swiss Webster mice to investigate the replication kinetics of TBEV/DEN4, TBEV/DEN4Δ30, or their mutant derivatives in mouse brain. Mice were inoculated IC with 10^3 PFU of virus and brains from at least three mice per group were harvested every other day, up to day 21, as described previously (Blaney et al., 2008; Pletnev et al., 2006). Mouse brains were individually homogenized as a 10% solution (w/v) using Hank's balanced salt solution (Invitrogen) supplemented with 7.5% sucrose, 5 mM sodium glutamate, 0.05 mg/ml ciprofloxacin (Bayer, Wayne, NJ), 0.06 mg/ml clindamycin (Pharmacia & Upjohn, New York, NY), and 0.0025 mg/ml amphotericin B (Quality Biologicals, Gaithersburg, MD). Brain suspensions were clarified by low-speed centrifugation and frozen at −80 °C until use. Virus titers in brain suspensions were quantitated by titration in Vero cells, as described above. Significant differences between viruses on each day were determined by unpaired *t* tests or one-way ANOVA followed by Tukey post hoc tests ($p<0.05$) (GraphPad Prism 5 software, La Jolla, CA). To investigate the stability of the engineered mutations within the chimeric viruses after their replication in the brain, virus RNA was extracted from brain homogenates obtained on the last days they were positive for virus and consensus sequences of the genomic regions encompassing the engineered mutations from each group were directly determined.

To investigate the neuroinvasive phenotype of TBEV/DEN4, TBEV/DEN4Δ30, and its derivatives, two sets of studies were performed in 3-week-old SCID mice (ICRSC-M; Taconic Farms). To measure neuroinvasiveness, 10 mice were inoculated IP with 10^5 PFU of TBEV/DEN4 virus, while separate groups of 33 to 56 mice were inoculated IP with 10^5 PFU of TBEV/DEN4Δ30 virus or its derivatives. Mice were observed for 49 days for signs of morbidity typical of CNS involvement, including paralysis. Moribund mice were humanely euthanized upon signs neurologic disease. Kaplan–Meier survival curves followed by Tukey post hoc tests were performed for statistical analysis ($p<0.05$) (GraphPad Prism 5 software). Separately, groups of 35 SCID mice were inoculated IP with 10^5 PFU of TBEV/DEN4 or TBEV/DEN4Δ30 virus, and the brains of three mice per group were harvested on odd days, for 21 days, to assess the level of virus replication. In addition, SCID mice in groups of 12 were inoculated IP with 10^5 PFU of TBEV/DEN4Δ30-derived mutant viruses. The brains of three mice from each of these groups were harvested on days 13, 15, 17, and 19 to assess the level of virus replication as described above. The significance of viral replication on each day was determined by unpaired *t* tests or one-way ANOVA followed by Tukey post hoc tests ($p<0.05$) (GraphPad Prism 5 software).

Histopathological analysis of brains of mice infected with mutant viruses

To investigate virus-induced pathology of the viruses in brains, 3-week-old female C57BL/6 mice (Taconic Farms) in groups of three were inoculated IC with 10^4 PFU of either TBEV/DEN4, TBEV/DEN4Δ30, or vΔ30/E_{315}/$NS5_{654,655}$, whereas three control mice were mock inoculated with Leibovitz's L-15 medium (Invitrogen). All mice were observed daily and euthanized on day 6, when TBEV/DEN4-infected mice developed paralysis. Mice were euthanized and perfused transcardially with PBS, and each mouse brain was dissected sagittally. The left hemisphere was frozen and stored at −80 °C for virus quantitation as described above. The right hemisphere was fixed in 4% paraformaldehyde for 72 h and processed according to standard histological methods. Twenty-five sections (30 μm thick) from each hemisphere were stained with hematoxylin and eosin (H&E) and analyzed for the presence and severity of virus-induced histopathology.

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