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Rapid Communication

Mutations in the NS2B and NS3 genes affect mouse neuroinvasiveness of a Western European field strain of tick-borne encephalitis virus

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Abstract

An attenuated strain (263) of the tick-borne encephalitis virus, isolated from field ticks, was either serially subcultured, 5 times in mice, or at 40 °C in PS cells, producing 2 independent strains, 263-m5 and 263-TR with identical genomes; both strains exhibited increased plaque size, neuroinvasiveness and temperature-resistance. Sequencing revealed two unique amino acid substitutions, one mapping close to the catalytic site of the viral protease. These observations imply that virus adaptation from ticks to mammals occurs by selection of pre-existing virulent variants from the quasispecies population rather than by the emergence of new random mutations. The significance of these observations is discussed.
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Keywords: Tick-borne encephalitis virus; Neuroinvasiveness; Viral protease

Introduction

Tick-borne encephalitis (TBE) is the most important arbovirus neuroinfection in Europe and Asia (Gritsun et al., 2003). It is caused by *Tick-borne encephalitis virus* (TBEV), a member of the genus *Flavivirus* within the family *Flaviviridae* (Thiel et al., 2005). In the Czech Republic, the incidence of TBE is one of the highest in Europe, with 400–1,000 clinical cases annually (according to data from EpiDat, www.szu.cz). However, serological surveys of the population in the southern region of the Czech Republic have revealed high seroprevalance with little clinical evidence of infection (Kopecký et al., 1991; Luňáčková et al., 2003). Thus, TBEV appears to infect a high proportion of humans relatively harmlessly and this needs to be explained. The virions are spherical particles, approximately 50 nm in diameter with a nucleocapsid composed of a (+) ssRNA genome enclosed in a capsid (C) protein and surrounded by a host cell-derived lipid bilayer. Two virus proteins are integrated in the lipid membrane, the envelope (E) protein and the membrane (M) protein. The (+)ssRNA genome is approximately 11 kb in length and contains one large open reading frame (ORF) which is flanked by 5' and 3' untranslated regions (UTR). The ORF encodes a single polyprotein that is cleaved by viral and cellular proteases into three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach and Rice, 2003). In general, wild-type strains of TBEV (i.e. field isolates) are neuropathogenic following either intracranial or peripheral inoculation and in the mouse model this usually results in lethal infections (Mandl, 2005). Sequencing of virus genomes and experiments with infectious clones have shown that TBEV neuropathogenicity is determined by mutations in different regions of the flavivirus genome, including those encoding virus

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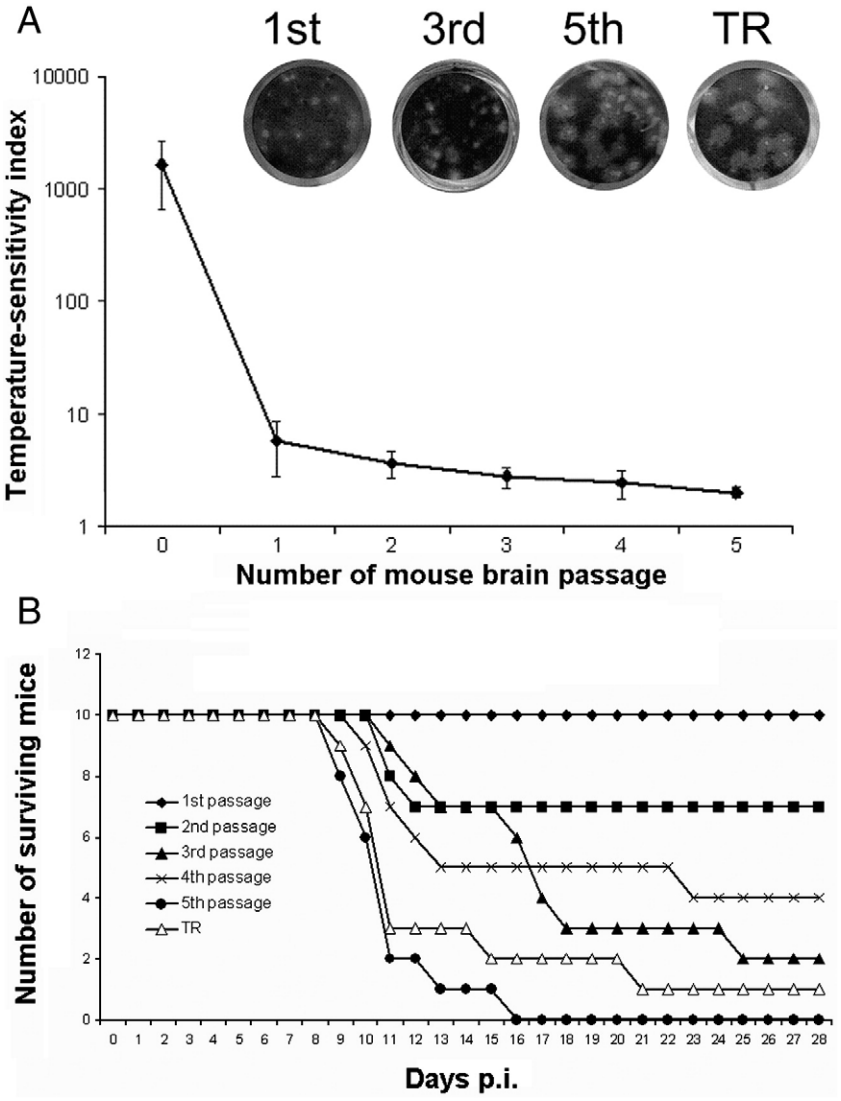


Fig. 1. Changes of biological properties of the strain 263 after subcultures in brains of suckling mice. (A) Loss of the temperature-sensitive phenotype of the strain 263 and change in the plaque morphology in PS cells after the 1st, 3rd and 5th passage in brains of suckling mice and comparison with the plaques produced by the strain 263-TR (TR). Temperature-sensitive index is expressed as the ratio of the titer (pfu) at the permissive (37 °C) and the non-permissive (40 °C) temperature (A). (B) Survival of adult mice subcutaneously inoculated with 100 pfu of the strain after consecutive passages in the brains of suckling mice and of the temperature-resistant variant of the strain 263-TR (TR).

proteins and untranslated regions (Hurrelbrink and McMinn, 2003). However most of the studied TBEV strains were subcultured many times in the laboratory and this is known to cause a shift of the phenotypic and genotypic characteristics of the virus (Dzhivanian et al., 1988; Kaluzová et al., 1994; Romanova et al., 2007). In the present work we subcultured natural isolates of a non-neuroinvasive low-passage strain 263, either 5 times in mice or in parallel once at 40 °C in PS cells, producing strains 263-m5 and 263-TR both of which proved to be more neuropathogenic in mice than the original 263 strain. As we shall argue later, further characterisation of these strains inferred that the rapid shift in virus neuropathogenicity that occurred during adaptation of the tick isolate to mammalian cells was due to the selection of virus quasispecies rather than to the emergence of new mutations. One of two identified mutations (NS3 A→S_{73}) mapped in close proximity to the NS2B–NS3 protease active site and may determine the neuro- or non-neuropathogenicity of the TBEV 263 strain. We propose that the non-virulent variants, found in the natural environment, are highly adapted to ticks and therefore might play an important role in sustaining human population immunity to TBEV by causing sub-clinical infections.

Results and discussion

It has been recognized since the 1960s, that TBEV isolated from field-caught ticks contains a heterogeneous population of variants that produce a range of plaque sizes, express temperature-sensitivity, and neuroinvasiveness (Mayer and Kožuch, 1969). Subclinical TBE infections were reported in monkeys and hamsters, but not in laboratory mice (Simon et al., 1966). It was therefore postulated that the wild-type strains of TBEV, i.e.

those isolated from the environment, are neuropathogenic following either intracranial or peripheral inoculation leading to lethal infections in mice (Mandl, 2005).

However, four strains (263, 274, 280, and 282) isolated from ticks in the Czech Republic are, a) completely non-neuroinvasive in laboratory mice, b) temperature-sensitive, c) turbid small plaque producers and d) slow growers in cell culture (Kopecký et al., 1991; Růžek et al., 2006). In this work we propagated strain 263 through suckling-mouse brains 5 times to study the stability of the non-neuroinvasive phenotype. After each passage, the basic biological properties of the virus (designated 263-m1, 263-m2, 263-m3, 263-m4 and 263-m5) were investigated, viz. temperature-sensitivity, plaque morphology and neuroinvasiveness. Reversion of temperature-sensitivity began to occur after the first passage and

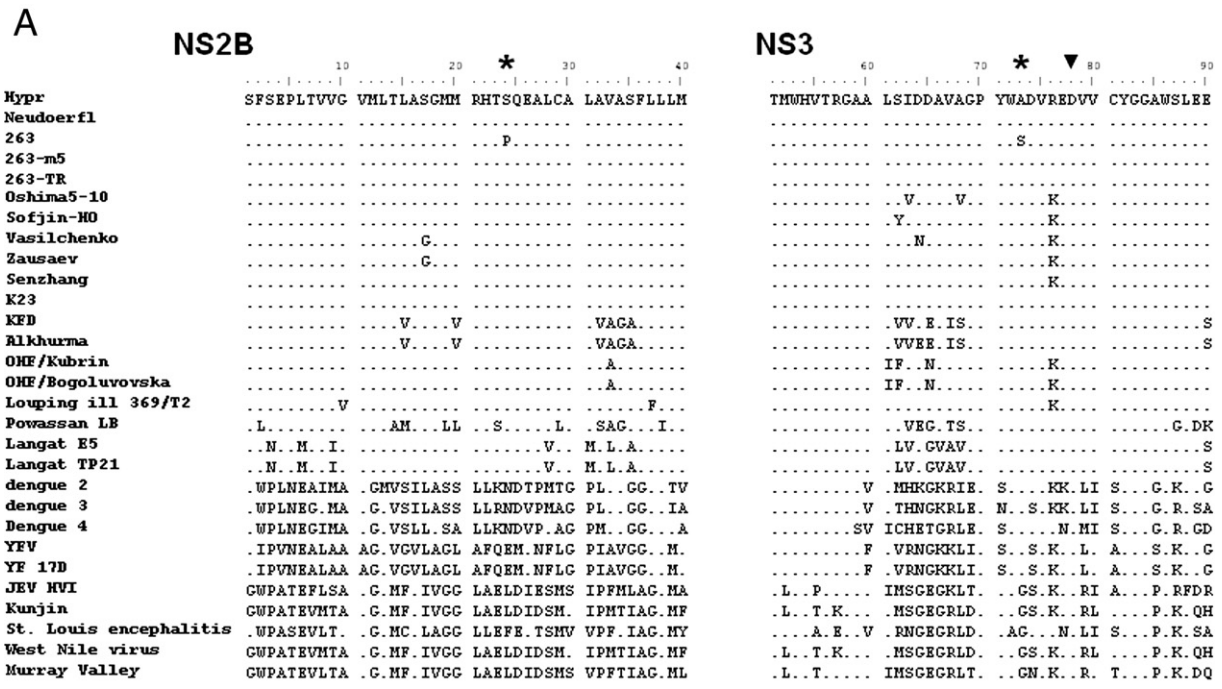
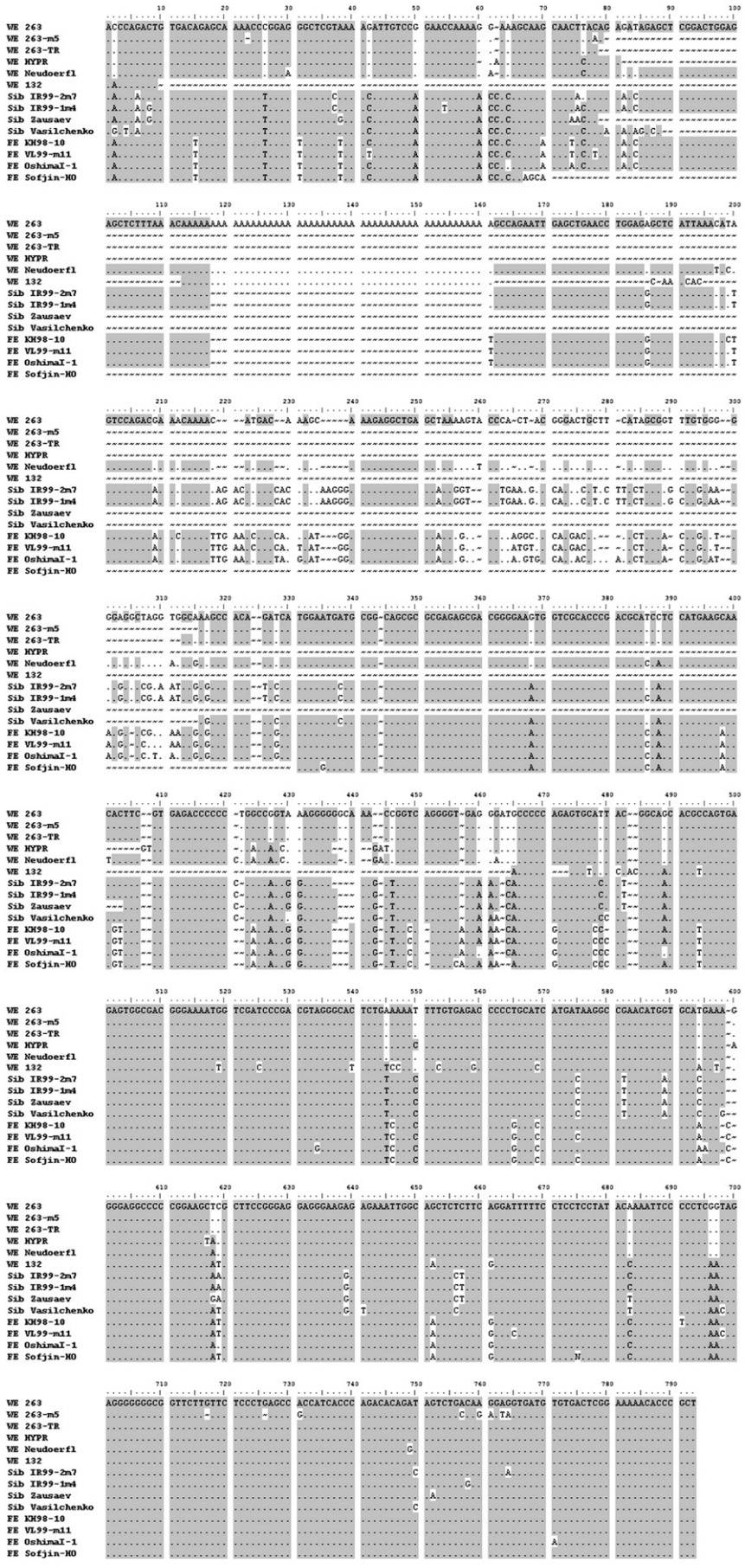


Fig. 2. Analysis of 263-derivative strains 263-m5 and 263-TR. (A) Aligned sequences of NS2B and NS3 proteins of selected flaviviruses. Positions 24 (NS2B) and 73 (NS3) are marked by asterisk and the catalytic amino acid Asp_{78} in NS3 by upside-down triangle. (B) Mapping of the mutation A→S_{73} of NS3 protein of 263 strain of TBEV on the 3D structure of WNV complex of NS3 with the central hydrophilic domain of NS2B (2ijo.pdb). Cartoon presentation created in Pymol. The N-terminal 180-amino acid domain of NS3 is presented as green ribbons and the NS2B hydrophilic domain between amino acids 50-96 is yellow. The catalytic site of the active serine protease is formed by the catalytic triad $His_{51}Asp_{75}Ser_{135}$ (red ribbons) and the loop of the NS2 protein is formed by the amino acids Asp_{82}-Phe_{85} (orange ribbons). The mutation Ala→Ser_{73} of the NS3 protein of the 263 strain of TBEV is highlighted in blue.



by the fifth passage, the titre of the 263-m5 recovered in cell cultures, was virtually the same at permissive and non-permissive temperatures (Fig. 1A). After the 3rd mouse passage, there was a mixture of small plaques (<1 mm in diameter) with a small proportion of large plaques (4–5 mm in diameter) but by the 5th passage in mice large plaques formed the predominant population (Fig. 1A). Similarly, neuroinvasiveness increased continuously with serial passage (Fig. 1B), and neuroinvasiveness of 263-m5 was virtually identical to that of the virulent Czech strain Hypr.

Independently of the serial passages in mice, the original temperature-sensitive virus (strain 263) was subcultured once at the non-permissive temperature (40 °C). This resulted in the selection of a temperature-resistant (263-TR) variant that produced titres at either 37 °C or 40 °C, differing by less than 10% pfu/ml and in contrast to the parental virus, was highly neuroinvasive for adult laboratory mice (Fig. 1B).

Whole genome sequence analysis revealed that 263-m5 and 263-TR were identical with each other at the amino acid level and differed from the original 263 isolate by two mutations that mapped at amino acid positions 24 and 73 of the NS2B and NS3 proteins respectively. To evaluate the significance of these substitutions a comparative alignment was constructed for the NS2B–NS3 region of all available flaviviruses. The NS3 protein in complex with the 47-amino acid central hydrophilic region of the NS2B protein functions as a serine protease responsible for the cleavage of virus polyprotein (Erbel et al., 2006). Alignment of 263 with other tick-borne flaviviruses revealed that 263-m5 and 263-TR displayed the "wild-type" TBEV genotype in relation to the NS2B S_{24} and NS3 A_{73} whereas NS2B P_{24} and NS3 Ser_{73} were unique amino acid substitutions of the non-neuroinvasive strain 263 (Fig. 2A). Mapping these mutations onto the available three-dimensional structure of the West Nile virus (WNV) serine protease complex NS2B–NS3 (Erbel et al., 2006) revealed that the conserved hydrophobic A_{73} (wild-type) of NS3 was substituted by hydrophilic S_{73} (of 263 virus). The NS3 $A \rightarrow S_{73}$ mutation (equivalent to the Gly_{70} of WNV) was localized in close proximity to a catalytic site that is formed by direct interaction of the catalytic triad $H_{51}D_{75}S_{135}$ of the NS3 protein with a loop produced by amino acids $D_{82}F_{85}$ of the NS2B protein (WNV enumeration). The A_{73} in the NS3 protein (TBEV enumeration) is absolutely conserved between all tick-borne and no-known vector flaviviruses (Fig. 2B). This might cause the reduction of NS2B–NS3 protease activity and also lead to the attenuation of the 263 strain. The second mutation $Ser_{24} \rightarrow Pro_{24}$, which is non-conserved, mapped in the hydrophobic region of the NS2B protein, outside the central hydrophilic domain which provides co-factor protease activity. It is unlikely that the $Ser_{24} \rightarrow Pro_{24}$ substitution directly influences the protease activity although it could cause instability of the NS2B–NS3 complex, especially at the non-permissive temperature. It is possible that the second mutation $Ser_{24} \rightarrow Pro_{24}$ of the 263 strain is "compensatory", i.e. without the first mutation, $A \rightarrow S_{73}$ may not be functional. Future experiments using infectious clones of TBEV may clarify these interpretations.

In comparison with strain 263, both 263-m5 and 263-TR, possessed a virtually identical large deletion in the proximal region of the 3'UTR (Fig. 3). The proximal 3'UTR domain was previously described as a variable domain (V3'UTR), located between the stop codon and terminal ~320-nucleotide region, conserved between tick-borne flaviviruses (C3'UTR or core). Neither spontaneous nor engineered deletions in the hypervariable domain affected virus pathogenicity in cell culture or mice (Mandl et al., 1998). One can therefore propose that this deletion did not contribute significantly to the pathogenetic properties of the virus.

Interestingly, despite the fact that both 263-TR and 263-m5 were derived independently, they displayed two identical amino acid differences and an almost identical deletion in the 3'UTR strongly suggesting that the selection of virus variants from a quasispecies population in strain 263, rather than the emergence of new mutants, occurred during laboratory passage of the parent virus in mice or PS cells. The likelihood of three identical independent mutations/deletions appearing in two distinct viruses after serial passage in mice or a single passage at a non-permissive temperature is much lower than the possibility of the selection of the same variant from quasispecies populations. It seems likely that a "wild-type" neuroinvasive (virulent) variant with NS2B-Ser_{24}/NS3-Ala_{73} was present as a minor quasispecies component within the non-neuroinvasive 263 virus population. One could then imagine that changing the host, from invertebrate (tick) to mammalian (mice or porcine kidney cells) resulted in preferential selection of the neuroinvasive variant. These two mutations in the NS2B and NS3 proteins were previously described in strain 263 amongst a total of 12 non-conserved unique amino acids, in comparison with two other neuroinvasive strains of Western European TBEV (Wallner et al., 1996).

In order to understand the difference in neuroinvasiveness between the neuroinvasive and non-neuroinvasive strains, we examined the distribution of these viruses in the tissues of adult mice at various time intervals. After subcutaneous inoculation of 100 pfu of strain 263, no virus was detected in the blood, or in any of the examined lymph nodes, spleen or brain at any of the monitored time points. However, the subcutaneous inoculation of 263-TR or 263-m5 led to efficient replication in all these organs (data not shown). The results suggest that attenuation is based on the inability of the virus to replicate in extraneural tissues and thus the virus is unable to induce viremia or to gain access to the central nervous system. Nevertheless, although the replication of strain 263 is restricted, the cellular and humoral, specific and nonspecific immune mechanisms are markedly activated in the murine host (J. Salát, unpublished results). A delay in virus spread from the site of inoculation is likely to attenuate neuroinvasiveness by allowing time for the host immune system to eliminate the infection (Hurrelbrink and McMinn, 2003).

Fig. 3. Sequence of the 3'UTR of the strain 263 and its derivates (263-m5 and 263-TR) aligned with other representatives of West-European (WE), Siberian (S) and Far Eastern (FE) subtype of TBEV (from the stop codon at position 10378 of the sequence of the European prototype strain Neudoerfl). GenBank accession numbers: Hypr (U39292), Neudoerfl (M77799), 132 (U27490), IR99-2m7 (AB049399), IR99-1m4 (AB049398), Zausaev (AF527415), Vasilchenko (L40361).

Virus transmission between ticks co-feeding on the same host in the absence of viremia was postulated to be the main mechanism of TBEV circulation in natural foci implying that viremia is a product, rather than a prerequisite, of efficient TBEV transmission (Labuda et al., 1997). TBEV can also be transmitted transovarially (Benda, 1958) and trans-sexually (Chunikhin et al., 1983). All of these transmission mechanisms suggest that most of the TBEV life-cycle is spent in ticks, rather than in mammalian hosts. A number of publications have shown that after mammal-to-tick transmission, a rapid shift in the phenotypic characteristics of TBEV occurs (Dzhivanian et al., 1988; Labuda et al., 1994; Kaluzová et al., 1994; Romanova et al., 2007). Serial passage of TBEV in *Hyalomma plumbeum* noticeably decreased the virulence for laboratory mice whereas subsequent virus passage through mouse brains led to restoration of the virulent phenotype (Dzhivanian et al., 1988). Similarly, passaging of TBEV through the salivary glands of *Ixodes ricinus* led to a reduction of neuroinvasiveness (Labuda et al., 1994) that was subsequently restored when the attenuated virus was passaged through mouse brains (Kaluzová et al., 1994). The selection, during serial passage, of pre-existing quasispecies was postulated to be the explanation for the rapid shift of virus phenotypic characteristics. Moreover, mutations in the E glycoprotein were thought to be responsible for the adaptation of TBEV to ticks or mammals (Labuda et al., 1994; Romanova et al., 2007). However in the present work we have demonstrated that selection of the more virulent strains 263-m5 and 263-TR occurred as the result of mutations in regions of the genome other than the E glycoprotein. Interestingly, with host-range mutants containing mutations either in the E protein (Labuda et al., 1994; Romanova et al., 2007) or the NS2B/NS3 protein (demonstrated here), viruses isolated from ticks, characteristically displayed reduced plaque size, reduced thermostability and reduced neuroinvasiveness. In contrast, strains adapted to mammals were rapidly-growing viruses, with large plaque morphology, increased thermostability and increased neuroinvasiveness. Whether or not virus growth characteristics rather than specificity of mutations (within E or NS2B/NS3) determine the host-range selection, remains to be determined.

The results imply that naturally occurring non-neuroinvasive TBEV strains circulate among virulent TBEV strains between ticks and can induce sub-clinical infections in humans and wild species. As revealed by the serological investigations, the ratio of manifest to unapparent human TBEV infections is approximately 2:3 (Luňáčková et al., 2003) indicating that a significant proportion of the human population in the Czech Republic may have been infected by attenuated non-neuroinvasive TBEV strains that thus sustain a high level of population immunity.

As far as we are aware, this is the first study to demonstrate, that virulent and attenuated viruses may co-exist as quasispecies in the same TBEV population and rapid conversion of neurovirulence during virus tick/mammal adaptation is mediated by selection from the quasispecies population rather than random mutagenesis during virus passage in the laboratory. Secondly, we have demonstrated that host-range adaptation is associated with mutations in the non-structural NS2B/NS3 proteins, with one mutation mapping close to the catalytic site of the virus serine protease. This contrasts with reports which identified host-range selection of variants with mutations in the E glycoprotein. These results extend our understanding of the molecular basis of TBEV circulation in natural foci and the induction of population immunity in endemic regions.

Material and methods

Viruses and cells

The Czech TBEV strain Hypr (accession number U39292) was used as a control. Hypr was originally isolated from the blood of a 10-year-old child diagnosed with tick-borne encephalitis in 1953 and has been propagated through numerous mouse brain passages. The complete genomic sequence (accession number U27491) of the studied strain 263 isolated from *I. ricinus* collected by flagging in 1987 (Kopecký et al., 1991; Růžek et al., 2006) was described previously (Wallner et al., 1996). The virus was propagated twice in suckling mice. Porcine kidney stable (PS) cells were grown at 37 °C in L-15 medium supplemented with 3% newborn calf serum and 1% mixture of Penicillin and Streptomycin (Sigma). Plaque morphology and virus titres were assayed on PS cell monolayers, as described previously (De Madrid and Porterfield, 1969).

Neuroinvasiveness testing

To estimate neuroinvasiveness, groups of 10 adult ICR mice (females, body weight 20–25 g; AnLab Prague, Czech Republic) were inoculated subcutaneously with 100 pfu of virus. Survival of mice was observed daily for a period of 28 days p.i.

Passaging of the virus in mouse brains

A group of suckling ICR mice (5 to 6 days old) was intracerebrally inoculated with 10^4 pfu of virus per mouse. During the acute stage of infection with apparent neurological signs of disease, the brains were removed, weighed, homogenized and prepared as a 20% suspension (w/v) in L-15 medium containing 3% newborn calf serum. Each homogenate was clarified by centrifugation at 5000 ×*g* and the supernatant medium was used for the inoculation of the next group of mice. After each passage, we investigated the basic biological properties of the virus: temperature-sensitivity, the character of the plaques in cell culture and neuroinvasiveness.

Virus growth in mouse tissues

Groups of adult ICR mice (females, body weight 20–25 g) were inoculated subcutaneously with 100 pfu of virus into the scruff of the neck. At the given time points after inoculation, 3 mice of each group were anesthetized and humanely killed. Specimens of the blood, lymph nodes (superficial cervical, axillary, brachial, and inguinal lymph nodes), spleen, and brain were collected. Organs were individually weighed and homogenized with a mortar and pestle, and prepared as 10 or 20% suspensions (w/v) in L-15 medium containing 3% newborn calf

serum. The suspensions were clarified by centrifugation at 10,000 ×*g* and supernatant fluids were titrated by plaque assay on PS cells. The detection thresholds were for specimens of blood 1.7 $\log_{10}$ pfu/ml, for 20% suspensions (brain, spleen) 2.4 $\log_{10}$ pfu/g, and for 10% suspensions (lymph nodes) 2.7 $\log_{10}$ pfu/g.

Sequence analysis

Nucleic acid sequences were determined by direct sequencing of both strands of PCR products with the automated Beckman CEQ 2000 DNA Analysis System (Beckman Coulter). The results of the sequence analysis were processed using Chromas, version 1.45 (http://www.techlysium.com.au/chromas.html). The nucleotide and deduced amino acid sequences were analyzed using BioEdit Sequence Alignment Editor, version 5.0.6. (http://www.mbio.nscu.edu/BioEdit/bioedit.html) and GeneDoc, version 2.6.002 (http://www.psc.edu/biomed/genedoc) and aligned with other available genomic sequences of TBEV strains. Alignments are available on request.

3D structure analysis of NS3 protein

The predicted three-dimensional structures of NS3 protein (2ijo.pdb) and the superimposition of mutations were produced using PYMOL facilities available at http://pymol.sourceforge.net/.

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