REVERTED VIRULENCE OF ATTENUATED TICK-BORNE ENCEPHALITIS VIRUS MUTANT IS NOT ACCOMPANIED WITH THE CHANGES IN DEDUCED VIRAL ENVELOPE PROTEIN AMINO ACID SEQUENCE

M. KALUZOVÁ, E. ELEČKOVÁ, E. ŽUFFOVÁ, J. PASTOREK, Š. KALUZ, O. KOŽUCH, M. LABUDA

Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 842 46 Bratislava, Slovak Republic

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Summary. – Serial passages of tick-borne encephalitis (TBE) virus strain 4387 isolated from the liver and lungs of the bank vole through the salivary glands of *Ixodes ricinus* ticks led to a reduction of its virulence for laboratory mice infected via peripheral route. When attenuated mutants were passaged through mouse brains, virulent phenotypes have appeared in the 3rd mouse passage. After 5 consecutive passages the virus was more pathogenic for mice after peripheral inoculation than the parental 4387 strain. The nucleotide sequence of the envelope proteins of the strain 4387 was studied after passaging through ticks salivary glands and subsequently through mice. The sequences coding for the envelope protein E of the virus from the first, third and fifth mouse passages were compared with those of parental virus and mutant attenuated in ticks.

The attenuated mutant differing from the parental strain 4387 by the amino acid substitution from glutamic acid to lysine at position 84, and from isoleucine to threonine at amino acid position 319 revealed strongly reduced pathogenicity for adult laboratory mice after peripheral inoculation. The attenuated mutant regained its virulence after 3 – 5 mouse brain passages, but the two amino acid substitutions were still conserved.

Key words: tick-borne encephalitis virus; attenuated and virulent strains; protein E; mutations

Introduction

TBE virus belongs to the family *Flaviviridae*, genus *Flavivirus*. Like other flaviviruses, TBE virus contains RNA genome of positive polarity with coding region for three structural proteins: the capsid (C) protein, the precursor of membrane (preM) protein, and the envelope (E) protein at the 5′ end. The coding region for the nonstructural proteins NS1 to NS5 continues towards the 3′ terminus of the genome. All viral proteins are encoded within a single open reading frame (Mandl *et al.*, 1988, 1989).

The flavivirus protein E contains about 500 amino acids and is usually glycosylated. It represents the viral haemagglutinin, induces a protective immune response and plays a central role in the biology of flaviviruses. Like other viral envelope proteins, protein E is believed to mediate also other important functions, such as receptor binding and the fusion of the virus membrane with cellular membranes after low pH-induced conformational changes (Guirakhoo *et al.*, 1989; Kimura *et al.*, 1986)

Virulence of an arbovirus is a complex character that is influenced by selective pressures of vertebrate hosts and artropod vectors (Nuttall *et al.*, 1991). The factors that influence the virulence of TBE virus are not yet defined but it seems reasonable to assume that the host through which a virus passes have a selective influence on the virus pheno- and genotype.

A single amino acid substition in E protein of TBE virus can lead to an attenuation as tested in the mouse model. By growing TBE virus in the presence of E protein – neutralizing antibodies, seven neutralization escape mutants were selected. One of the mutants with amino acid substitution from tyrosine to histidine at position 384 in domain B of the E protein revealed strongly reduced pathogenicity for adult mice after peripheral inoculation, but retained its capacity to induce high titers of antibodies (Holzmann *et al.*, 1990).

Similar experiments were done with Sarawak strain of Japanese encephalitis virus. Eight neutralization escape mutants of E protein were obtained. Several showed decreased virulence in 3 week-old mice after intraperitoneal inocula-

tion and two of them showed reduced virulence in 2 week-old mice inoculated intracranially (ic). One of these mutants induced protective immunity in adult mice against challenge with parental virus. Nucleotide sequencing of the E coding region of the mutant revealed single base change resulting in substitution from isoleucine to serine at amino acid position 270 (Cecilia and Gould, 1991).

Serial passages of TBE virus strain 4387 isolated from the liver and lungs of a bank vole (Kožuch *et al.*, 1990) in *Ixodes ricinus* ticks, were accompanied by gradual reduction in virulence of the virus as indicated by transmission of virus by infected ticks feeding on laboratory mice. After the 7th tick passage (strain 4387/Ir7), 95% of mice survived the bite of infected ticks and the same virus was avirulent also after subcutaneous (sc) inoculation of laboratory mice (Labuda *et al.*, 1994).

Nucleotide sequencing of the protein E gene of the virulent strain 4387 showed 3 amino acid codon differences from TBE virus strain Neudorfl. The attenuated virus 4387/Ir7 had two additional amino acid substitutions, one which was different from both parental and Neudorfl TBE viruses (glutamic acid to lysine at position 84) and a second substitution that was identical to Neudorfl virus (isoleucine to threonine at position 319) (Labuda *et al.*, 1994).

In this study, the phenotypic changes in the tick-passaged TBE virus mutant 4387/Ir7 were tested after sequences in central nervous system of laboratory mice. The fragments coding for protein E of selected mutants were characterized after serial passages in the vertebrate host.

Materials and Methods

Viruses. TBE virus strain 4387 was isolated from liver and lungs of a female bank vole (*Clethrionomys glareolus*) trapped in Gbelce (southern Slovakia) (Kožuch *et al.*, 1990). Virus stocks were prepared as 10% (w/v) suspension of infected suckling mouse brains in Eagles's Minimal Medium supplemented with 10% foetal bovine serum and antibiotics. Virus titers of parent isolate 4387 in 15 g laboratory mice were 10^7 ic LD_{50}/0.03 ml and 10^6 sc LD_{50}/0.05 ml.

Virus passages and virus assay. TBE virus mutant 4387/Ir7 underwent passages in suckling laboratory mouse brains by ic inoculation of 0.01 ml of mouse brain-derived virus material. Virus stocks (1st, 3rd, and 5th mouse brain passage) were titrated in suckling mice by the ic route inoculating 0.01 ml of virus material and in adult (15 g) laboratory mice by the sc route inoculating 0.05 ml of virus material. Surviving mice were bled on day 21 p.i. for serological examination and on the next day challenged with 1 000 ic LD_{50} in 0.05 ml of virulent TBE virus strain Hypr.

Haemagglutination-inhibition (HI) test. Sera of mice were extracted by acetone and adsorbed with goose erythrocytes prior testing for the presence of HI antibodies. The antigens for the HI tests were prepared by sucrose-acetone extraction of suc mouse brains according to Clarke and Casals (1958). Four to haemagglutinating units of antigen were used in the tests.

Nucleotide sequencing of protein E gene of TBE viruses. cellular RNA was prepared from infected suckling mouse suspensions using guanidinium thiocyanate (Chomczynsk Sacchi, 1987). 2 µg of total cellular RNA with 700 ng of anti primer TCGACTCCAAGGGTCATGGCCA correspondi the 3′ end of gene coding protein E (nucleotides 2415 – Mandl *et al.*, 1988) was heat denatured at 70 °C for 10 min coded on ice. Reverse transcription was performed in a volume of 20 µl containing 50 mmol/l Tris-HCl, pH 50 mmol/l KCl, 10 mmol/l $MgCl_2$, 10 mmol/l DTT, 28 U sin (Promega), 1 mmol/l of each deoxynucleoside triphosp and 20 U Moloney murine reverse transcriptase (Pharmacia) mixture was incubated for 1 hr at 37 °C, then for 5 mins at and cooled on ice.

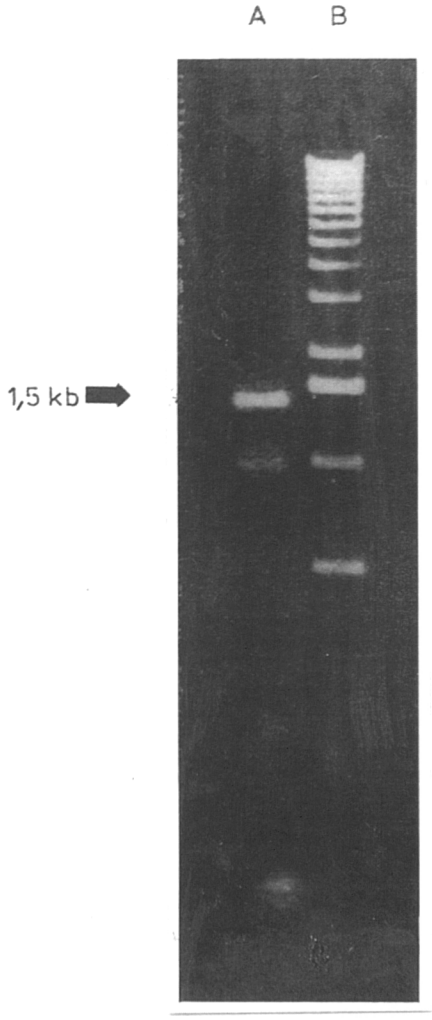

Fig. 1
Agarose gel electrophoresis of the produkt of reverse transcri polymerase chain reaction
Total RNA was isolated from TBE virus 4387/Ir7 in its 1st mouse passage (A). 1kb DNA ladder was used as a marker (B).

Then 6 µl of this reaction mixture was used in the subsequent amplification, which was performed in a final volume of 50 µl containing 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.1% gelatin, 200 ng of sense primer (nucleotides 953 – 972, GATCGCGTTGCACACACTTGGA), 200 ng of antisense primer (the same as in cDNA synthesis), 0.2 mmol/l of each deoxynucleoside triphosphate and 2.5 U Taq DNA polymerase (Promega). The mixture was overlaid with 50 µl of mineral oil. The amplification was performed in an automated thermal cycler (Pharmacia LKB Gene ATAQ Controller) at 95 °C for 5 mins and in 40 cycles at 94 °C for 1 min, at 58 °C for 1.15 min and at 72 °C for 1.50 min. The final extension was at 72 °C for 10 mins. The PCR product of the expected length after 1st (Fig. 1), 3rd and 5th mouse brain passage was electrophoresed in 1% agarose-ethidium bromide gel in TBE buffer.There was no difference between the tested passages. Fragments were purified from agarose gel by Gene Clean (BIO 101).

The PCR product was treated with 5 U T4 polynucleotide kinase (BRL Gibco), and blunt ends were made with 2.5 U Klenow fragment DNA polymerase I (Boehringer) and 0.025 mmol/l of each deoxynucleoside triphosphate. The ligation was performed in 30 µl volume using 200 ng *Eco*RV-cut pBluescript KS$^-$ (Stratagene), 66 mmol/l Tris-HCl pH 7.6, 6.6 mmol/l $MgCl_2$, 10 mmol/l DTT, 66 µM ATP, 5% polyethylene glycol, and 1 U T4 DNA ligase (Boehringer) at room temperature for 4 hrs. To the ligation mixture was added NaCl 0.18 mol/l, digested with 2.5 U *Eco*RV (Boehringer) and used for transforming of *Escherichia coli* DH5alphaMCR (Gibco, BRL) utilizing white-blue X-gal and IPTG (Boehringer) selection.

Putative positive clones were selected by quick screen method comparing mobility of covalently closed circular forms of plasmids. After digestion with *Sal*I and *Xba*I (Boehringer) positive clones containing the fragment of expected size were chosen and then sequencing (T7 sequencing kit, Pharmacia) of this clone was carried out by the dideoxy-method of Sanger *et al.* (1977), using double-stranded template and gene specific primers. Clones were sequenced in duplicates.

Results

The change in the phenotype of the attenuated TBE virus mutant 4387/Ir7 after passaging in CNS of laboratory mice

When attenuated TBE virus mutant 4387/Ir7 obtained by serial passages in *I. ricinus* ticks was inoculated ic into suckling mice and the mouse brain derived virus was used for sc inoculation of adult mice, the virus mutant retained its attenuation. All mice inoculated either with 10% mouse brain suspension (10^8 ic LD_{50}/0.01 ml) or its tenfold dilutions up to 1 LD_{50}/0.01 ml survived the infection, developed HI antibodies in titers 1:40 – 1:640 and were protected against the challege with the virulent TBE virus (strain Hypr) in 100% when the titers of HI antibodies were higher than 1:40. The mice with titers of HI antibodies 1:40 (50% of mice inoculated with 1 LD_{50}) survived the challege in 50%.

Virulent phenotype of TBE virus mutant 4387/Ir7 reappeared in the 3rd mouse brain passage. From 84 adult mice inoculated with virus doses 10^8 – 10^2 ic LD_{50} (79%) died. The 18 surviving mice (21%) developed HI Ab in titres higher than 1:40 and survived the challenge with virulent TBE virus. Fifty percent of mice infected with 1 ic LD_{50} survived and 50% of these survived the challenge (Table 1).

Table 1. Changes in the phenotype of TBE virus strain 4387/Ir7 after passaging in CNS of mice

First virus dose (ic LD_{50}/0.05 ml)	Mice surviving/inoculated (%)			
	The 3rd passage virus		The 5th passage virus	
	Infection	Challenge	Infection	Challenge
10^8	1/12 (9%)	1/1	1/12 (9%)	1/1
10^7	4/12 (33%)	4/4	1/12 (9%)	1/1
10^6	1/12 (9%)	1/1	0/12 (9%)	–
10^5	4/12 (33%)	4/4	1/12 (9%)	1/1
10^4	3/12 (25%)	3/3	0/12 (0%)	–
10^3	2/12 (17%)	2/2	0/12 (0%)	–
10^2	3/12 (25%)	3/3	0/12 (0%)	–
10^1	6/12 (50%)	3/6	2/12 (17%)	2/2
10^0	12/12(100%)	3/12	6/12 (50%)	3/6

Mice infected sc with various doses of TBE virus strain 4387/Ir7 from the 3rd and 5th mouse brain passage. Mice subsequently challenged sc with 1 000 LD_{50}/0.05 ml of TBE virus strain Hypr.

After two additional mouse brain passages the virus titer in suckling mice reached 10^{10} ic LD_{50}/0.03 ml and the virus was more pathogenic for adult mice than the parental strain 4387 after peripheral inoculation, but still some mice (9%) survived the sc inoculation of high doses of virus (10^8 – 10^5 ic LD_{50}), developed HI antibodies in high titers (1:640 – 1:1280), and survived the challenge. After the inoculation of mice with 10 and 1 ic LD_{50} survived 17%, and 50% of animals, respectively. Half of the mice in inoculated with the lowest virus doses did not develop sufficiently high HI antibodies to survive the challenge (Table 1). The calculated titer of this virus passage ($10^{8.8}$ sc LD_{50}/0.05ml) was by 2.8 log units higher than that of the parental virus strain 4387.

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                S  R  C   T  H  L  E   N  R  D   F  V  T   G  T  Q  G
Neu  1      UCGCGUUGCA CACACUUGGA AAACAGGGAC UUUGUGACUG GUACUCAGGG
4387/Ir7M1       ..... .......... .......... .......... ..........
4387/Ir7M3       ..... .......... .......... .......... ..........
4387/Ir7M5  ......... .......... .......... .......... ..........

                  T  T  R   V  T  L   V  L  E  L   G  G  C   V  T  I
Neu    51   GACUACGAGG GUCACCUUGG UGCUGGAACU GGGUGGAUGU GUUACUAUAA
4387/Ir7M1  .......... .......... ....A..... .......... .....C....
4387/Ir7M3  .......... .......... ....A..... .......... .....C....
4387/Ir7M5  .......... .......... ....A..... .......... .....C....

               I  A  E  G   K  T  S   M  D  V   W  L  D  A   I  Y  Q
Neu    101  CAGCUGAGGG GAAGCCUUCA AUGGAUGUGU GGCUUGACGC CAUUUACCAG
4387/Ir7M1  .......... ...A...... .......... .......... ..........
4387/Ir7M3  .......... ...A...... .......... .......... ..........
4387/Ir7M5  .......... ...A...... .......... .......... ..........

                E  N  P   A  K  T  R   E  Y  C   L  H  A   K  L  S  D
Neu    151  GAGAACCCUG CUAAGACACG UGAGUACUGU UUACACGCCA AGUUGUCGGA
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5  .......... .......... .......... .......... ..........

                  T  K  V   A  A  R   C  P  T  M   G  P  A   T  L  A
Neu    201  CACUAAGGUU GCAGCCAGAU GCCCAACAAU GGGACCAGCC ACUUUGGCUG
4387/Ir7M1  .......... .......... .......G.. .......... .........A
4387/Ir7M3  .......... .......... .......G.. .......... .........A
4387/Ir7M5  .......... .......... .......G.. .......... .........A

               E  E  H  Q   G  G  T   V  C  K   R  D  Q  S   D  R  G
Neu    251  AAGAACACCA GGGUGGCACA GUGUGUAAGA GAGAUCAGAG UGAUCGAGGC
4387/Ir7M1  .......... ...C...... .......... .......... ...C......
4387/Ir7M3  .......... ...C...... .......... .......... ...C......
4387/Ir7M5  .......... ...C...... .......... .......... ...C......
            K

                W  G  N   H  C  G  L   F  G  K   G  S  I   V  A  C  V
Neu    301  UGGGGCAACC ACUGUGGACU GUUUGGAAAG GGUAGCAUUG UGGCCUGUGU
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5  .......... .......... .......... .......... ..........

                  K  A  A   C  E  A   K  K  K  A   T  G  H   V  Y  D
Neu    351  CAAGGCGGCU UGUGAGGCAA AAAAGAAAGC CACAGGACAU GUGUACGACG
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5  .......... .......... .......... .......... ..........

                A  N  K  I   V  Y  T   V  K  V   E  P  H  T   G  D  Y
Neu    401  CCAACAAAAU AGUGUACACG GUCAAAGUCG AACCACACAC GGGAGACUAU
4387/Ir7M1  .......... .......... ..U....... .......... ..........
4387/Ir7M3  .......... .......... ..U....... .......... ..........
4387/Ir7M5  .......... .......... ..U....... .......... ..........
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Fig. 2

For legend see page 139.

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                V  A  A   N  E  T  H   S  G  R   K  T  A   S  F  T  I
Neu     451 GUUGCCGCAA ACGAGACACA UAGUGGGAGG AAGACGGCAU CCUUCACAAU
4387/Ir7M1  .......... .......... .......... .......... .........G.
4387/Ir7M3  .......... .......... .......... .......... .........G.
4387/Ir7M5  .......... .......... .......... .......... .........G.
                                                                  V

                S  S  E   K  T  I   L  T  M  G   E  Y  G   D  V  S
Neu     501 UUCUUCAGAG AAAACCAUUU UGACUAUGGG UGAGUAUGGA GAUGUGUCUU
4387/Ir7M1  .......... .........C ....U..... .......... ..........
4387/Ir7M3  .......... .........C ....U..... .......... ..........
4387/Ir7M5  .......... .........C ....U..... .....

            L  L  C  R   V  A  S   G  V  D   L  A  Q  T   V  I  L
Neu     551 UGUUGUGCAG GGUCGCUAGU GGCGUUGACU UGGCCCAGAC CGUCAUCCUU
4387/Ir7M1  .......... .......... ..U.......
4387/Ir7M3  .......... .......... ..U....... .......... ..........
4387/Ir7M5

               E  L  D   K  T  V  E   H  L  P   T  A  W   Q  V  H  R
Neu     601 GAGCUUGACA AGACAGUGGA ACACCUUCCA ACGGCUUGGC AGGUCCAUAG
4387/Ir7M1                                                        ..
4387/Ir7M3  .......... .......... .......... .......... .......C..
4387/Ir7M5

               D  W  F   N  D  L   A  L  P  W   K  H  E   G  A  Q
Neu     651 GGACUGGUUC AAUGAUCUGG CUCUGCCAUG GAAACAUGAG GGAGCGCAAA
4387/Ir7M1  .......... ....
4387/Ir7M3  .......... .......... .....
4387/Ir7M5

            N  W  N  N   A  E  R   L  V  E   F  G  A  P   H  A  V
Neu     701 ACUGGAACAA CGCAGAAAGA CUGGUUGAAU UUGGGGCUCC UCACGCUGUC
4387/Ir7M1
4387/Ir7M3  .......U.. ..
4387/Ir7M5

               K  M  D   V  Y  N  L   G  D  Q   T  G  V   L  L  K  A
Neu     751 AAGAUGGACG UGUACAACCU CGGAGACCAG ACUGGAGUGU UACUGAAGGC
4387/Ir7M1                    ... .......... .......... ..........
4387/Ir7M3                    ... .......... .......... ..........
4387/Ir7M5                      . .......... .......... ..........

               L  A  G   V  P  V   A  H  I  E   G  T  K   Y  H  L
Neu     801 UCUCGCUGGG GUUCCUGUGG CACACAUUGA GGGAACCAAG UACCACCUGA
4387/Ir7M1  .......... .....C.... .......... .......... ..........
4387/Ir7M3  .......... .....C.... .......... .......... ..........
4387/Ir7M5  .......... .....C.... .......... .......... ..........

            K  S  G  H   V  T  C   E  V  G   L  E  K  L   K  M  K
Neu     851 AGAGUGGCCA CGUGACCUGC GAAGUGGGAC UGGAAAAACU GAAGAUGAAA
4387/Ir7M1  .......... U.....A... .......... .......... ..........
4387/Ir7M3  .......... U.....A... .......... .......... ..........
4387/Ir7M5  .......... U.....A... .......... .......... ..........
```

Fig. 2

For legend see page 139.

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                G  L  T    Y  T  M  C    D  K  T    K  F  T    W  K  R  A
Neu     901 GGUCUUACGU ACACAAUGUG UGACAAAACA AAGUUCACAU GGAAGAGAGC
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5  .......... .......... .......... .......... ..........

                P  T  D    S  G  H    D  T  V  V    M  E  V    T  F  S
Neu     951 UCCAACAGAC AGUGGGCAUG AUACAGUGGU CAUGGAAGUC ACAUUCUCUG
4387/Ir7M1  .......... .......... .C........ .......... ..........
4387/Ir7M3  .......... .......... .C........ .......... ..........
4387/Ir7M5  .......... .....

                G  T  K  P    C  R  I    P  V  R    A  V  A  H    G  S  P
Neu    1001 GAACAAAGCC CUGUAGGAUC CCAGUCAGGG CAGUGGCACA UGGAUCUCCA
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5

                D  V  N    V  A  M  L    I  T  P    N  P  T    I  E  N  N
Neu    1051 GAUGUGAACG UGGCCAUGCU GAUAACGCCA AACCCAACAA UUGAAAACAA
4387/Ir7M1  .......... .......... .......... .......... ......C...
4387/Ir7M3  .......... .......... .......... .......... ......C...
4387/Ir7M5                                                       T

                G  G  G    F  I  E    M  Q  L  P    P  G  D    N  I  I
Neu    1101 UGGAGGUGGC UUCAUAGAGA UGCAGCUGCC CCCAGGGGAU AACAUCAUCU
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5

                Y  V  G  E    L  S  H    Q  W  F    Q  K  G  S    S  I  G
Neu    1151 AUGUUGGGGA ACUGAGUCAU CAAUGGUUCC AAAAAGGGAG CAGCAUCGGA
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5

                R  V  F    Q  K  T  K    K  G  I    E  R  L    T  V  I  G
Neu    1201 AGGGUUUUCC AAAAGACCAA GAAAGGCAUA GAAAGACUGA CAGUGAUAGG
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5

                E  H  A    W  D  F    G  S  A  G    G  F  L    S  S  I
Neu    1251 AGAGCACGCC UGGGACUUCG GUUCUGCUGG AGGCUUUCUG AGUUCAAUUG
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5

                G  K  A  V    H  T  V    L  G  G    A  F  N  S    I  F  G
Neu    1301 GGAAGGCGGU ACAUACGGUC CUUGGUGGCG CUUUCAACAG CAUCUUCGGG
4387/Ir7M1  .......... G......... ........U. .......... ..........
4387/Ir7M3  .......... G......... ........U. .......... ..........
4387/Ir7M5
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Fig. 2

For legend see page 139.

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                G  V  G   F  L  P  K   L  L  L   G  V  A   L  A  W  L
Neu    1351 GGAGUGGGGU UUCUACCAAA ACUUUUAUUA GGAGUGGCAU UGGCUUGGUU
4387/Ir7M1  .......... .......... G......... .......... ..........
4387/Ir7M3  .......... .......... G......... .......... ..........
4387/Ir7M5

                G  L  N   M  R  N   P  T  M  S   M  S  F   L  L  A
Neu    1401 GGGCCUGAAC AUGAGAAACC CUACAAUGUC CAUGAGCUUU CUCUUGGCUG
4387/Ir7M1  .......... .......... .......... .......... ..........
4387/Ir7M3  .......... .......... .......... .......... ..........
4387/Ir7M5

                G  G  L  V   L  A  M   T  L  G   V  G  A
Neu    1451 GAGGUCUGGU CUUGGCCAUG ACCCUUGGAG UGGGGGCG
4387/Ir7M1  .......... .......... .......... .
4387/Ir7M3  .......... .......... ....
4387/Ir7M5
```

Fig. 2

Nucleotide and deduced amino acid sequences of protein E gene of TBE viruses

The first, third and fifth passage of mutant 4387/Ir7 (M1,M3,M5) through mice. Strain Neudorfl (Neu). The regions of interest were sequenced only.

Comparison of the nucleotide and deduced amino acid sequences of the E protein gene of TBE virus strain 4387/Ir7 after mouse brain passage

Comparing the sequence coding for protein E of TBE virus strain 4387/Ir7 passaged through mice with that of the source virus strain 4387/Ir7 did not show any difference, but it differed from parental strain 4387 at nucleotide position:

228:A->G transition, silent mutation

250:G->A transition, amino acid mutation Glu->Lys

956:U->C transition, amino acid mutation Ile->Thr

The parental strain 4387 differed from the prototype strain Neudorfl by 3 amino acid mutations and several silent changes in protein E (Fig. 2).

The attenuated TBE virus mutant, which differed from the parental strain 4387 by amino acid substitutions from glutamic acid to lysine at amino acid position 84 and from isoleucine to threonine at amino acid position 319 revealed reduced pathogenicity after peripheral inoculation of adult laboratory mice. But in the 3rd and 5th mouse brain passages this substitution was still conserved and the virulence increased after peripheral inoculation.

Discussion

The experiments, in which attenuated TBE virus was selected by passages in *Hyalomma plumbeum* ticks and virulent variant appeared after repassages in laboratory mice were done by Dzivanyan *et al.* (1988). Our experiments (Labuda *et al.*, 1994) have shown a sequential decrease in virulence of strain 4387 for mice, when passaged serially through *I.ricinus* ticks. These observations suggested that strain 4387 was a heterogeneous pool of variants with different relative virulence for mice. Presumably, avirulent variant was selected from parental TBE virus strain 4387 under selective pressure. The nucleotide sequencing data (Labuda *et al.*, 1994) showed that low virulent passaged strain 4387/Ir7 had 3 nucleotide differences in the gene encoding E protein as compared with the parental virus from which it was derived and 2 of them led to amino acid changes. Surprisingly, these changes were conserved during passages through laboratory mice, when the virulence reappeared. In the process of reappearence of virulence the virus differed markedly from the parental virus 4387, namely it did not respond in the ussual manner to the dilution and mice frequently survived high virus doses.

Apparently, the mutations in the virus genome region coding for protein E were not responsible for this phenotype. As regards the mutation from isoleucine to threonine at position 319, it altered only the nature of the amino acid residue from nonpolar to polar. This change was in the variable region of protein E. The prototype strain Neudorfl has also threonine at this position (Mandl *et al.*, 1988).

It is interesting to note that at amino acid position 84 in E protein, glutamic acid is found in all yellow fever viruses so far sequenced, including live vaccine 17D (Jennings *et al.*, 1993), in dengue viruses and in the complex of TBE viruses represented by Western subtype, Far eastern subtype of TBE virus, Neigishi, Langat and Louping ill viruses (Mandl *et al.*, 1988; Pletnev *et al.*, 1990; Deubel *et al.*, 1988).

However, lysine is found at this amino acid position in E proteins of all neurotropic flaviviruses so far studied, including West Nile, St. Luis encephalitis and several strains of Murray Valley encephalitis (Castle *et al.*, 1986; Trent *et al.*, 1987; Lobigs *et al.*, 1988; Nitayaphan 1990). Significance of amino acid mutations at position 84 from glutamic acid to lysine and at position 319 from isoleucine to threonine in protein E is not fully understood. E.g. yellow fever 17D vaccine strain contains a total of 68 nucleotide changes, also in other proteins, or even in noncoding sequences of the genome (12 amino acid changes), several of which may contribute to the attenuation (Jennings *et al.*, 1992).

It is possible that under the selective pressure of a factor in salivary glands of ticks the attenuated virus was obtained from heterologous population, and in the process of "readaptation" to the mouse brain the virus regained its virulence by changes in other genome regions. To provide a more complex picture and to understand better the relative role of different parts of the genome in the TBE virus virulence, also other genes of virus genome should be investigated.

References

Castle, E., Leidner, U., Nowak, T., Wengler, G., and Wengler, G. (1986): Primary structure of the West Nile flavivirus genome region coding for all nonstructural proteins. *Virology* **149**, 10–26.

Cecilia, D., and Gould, E. A. (1991): Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-rezistant mutants. *Virology* **181**, 70–77.

Clarke, D., and Casals, J. (1958): Techniques for haemagglutination and haemagglutination-inhibition with arthropod-borne viruses. *Am. J. trop. Med. Hyg.* **7**, 561–573.

Chomczynski, P., and Sacchi, N. (1987): Single-step method of RNA isolation by acid quanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Deubel, V., Kinney, R. M., and Trent, D. W. (1988): Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica genotype: Comparative analysis of the full-length genome. *Virology* **165**, 234–244.

Dzivanyan, T. I., Korolev, M. B., and Kaganov, G. G. (1988): Changes in hosts dependent characteristics of tick-borne encephalitis virus during its adaptation to ticks and readaptation to white mice. *Vopr. Virusol.* **5**, 589–595.

Guirakhoo, F., Heinz, F. X., and Kunz, C. (1989): Epitope model of tick-borne encephalitis virus envelope glycoprotein E: analysis of structural properties, role of carbohydrate side chain, and conformational changes occuring at acidic pH. *Virology* **169**, 90–99.

Holzmann, H., Heinz, F. X., Mandl, C. W., Guirakhoo, F., and Kunz, C. (1990): A single amino acid substitution in envelope protein E of tick-borne encephalitis virus leds to attenuation in the mouse model. *J. Virol.* **64**, 5156–5159.

Jennings, A. D., Whitby, J. E., Minor, P. D., and Barrett, A. D. T. (1993): Comparison of the nucleotide and deduced amino acid sequences of the envelope protein genes of the wild- type French viscerotropic strain of yellow fever virus and the live vaccine strain, French neurotropic vaccine, derived from it. *Virology* **192**, 692–695.

Kimura, T., Gollins, S. W., and Porterfield, J. S. (1986): The effect of pH on the early interactions of West Nile virus with P388D1 cells. *J. gen. Virol.* **67**, 2423–2433.

Kožuch, O., Labuda, M., Lysý, J., Weismann, P., and Krippel, E. (1990): Longitudinal study of natural foci of Central European encephalitis virus in West Slovakia. *Acta virol.* **34**, 537–544.

Labuda, M., Jiang, W.R., Kaluzová, M., Kožuch, O., Nuttall, P.A., Weismann, P., Elečková, E., Žuffová, E., and Gould, E.A (1994): Change in phenotype of tick-borne encephalitis virus following passage in *Ixodes ricinus* ticks and associated amino acid substitution in the envelope protein. *Virus Res.* **31**, 305–315.

Lobigs, M., Marshall, I. D., Wier, R. C., and Dalgarno, L. (1988): Murray Valley encephalitis virus field strains from Australia and Papua New Guinea, studies on sequence of the major envelope protein gene and virulence for mice. *Virology* **165**, 245–255.

Mandl, C. W., Heinz, F. X., and Kunz, C. (1988): Sequence of the structural proteins of tick borne encephalitis virus (Western subtype) and comparative analysis with other flaviviruses. *Virology* **166**, 197–205.

Mandl, C. W., Heinz, F. X., Söckl, E., and Kunz, C. (1989): Genome sequence of tick-borne encephalitis (Western subtype) and comparative analysis of nonstructural proteins with other flaviviruses. *Virology* **173**, 291–301.

Nitayaphan, S., Grant, J. A., Chang, G.- J. J., and Trent, D. W. (1990): Nucleotide sequence of virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivate SA-14-14-2. *Virology* **177**, 541–552.

Nuttal, P. A., Jones, L. D., and Davies, C. R. (1991): The role of arthropod vectors in arbovirus evolution. *Advances in Disease Vector Research* **8**, 15–45.

Pletnev, A. G., Yamshchikov, V. F., and Blinov, V. M. (1990): Nucleotide sequence of the genome and complete amino acid sequence of the polyprotein of tick-borne encephalitis virus. *Virology* **174**, 250–263.

Trent, D. W., Kinney, R. M., Johnson, B. J. B., Vance-Vorndan, A., Grant, J. A., Deubel, V., Rice, C. M., and Hahn, C. (1987): Partial nucleotide sequence of St. Luis encephalitis virus RNA, structural proteins NS1, ns2a, ns2b. *Virology* **156**, 293–304.

Sanger, F. Nicklen, S. and Coulson, A. R. (1977): DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Westaway, E. G., Brinton, M. A., Gaidamovich, S. Y., Horzinek, M. C., Igarashi, A ., Kaariainen, L., Lvov, D. K., Portefield, J. S., Russell, P. K., and Trent, D. W. (1985): Flaviviridae. *Intervirology* **24**, 183–190.