ANTIGENIC SIMILARITY OF CENTRAL EUROPEAN ENCEPHALITIS AND LOUPING-ILL VIRUSES

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Summary. – Twenty isolates of Central European encephalitis (CEE) virus were compared with 20 isolates of louping-ill (LI) virus in indirect immunofluorescence test (IIFT), using a panel of 17 monoclonal antibodies (MoAbs) prepared against the prototype LI virus. Three Asian members of the tick-borne encephalitis (TBE) complex were also included in the comparison: Turkish sheep encephalitis (TSE), Russian spring-summer encephalitis (RSSE) and Langat (LGT) viruses. Antigenic relationships of the viruses were evaluated by Dice similarity coefficient and cluster analysis. The results revealed antigenic heterogeneity of LI isolates, antigenic homogeneity of CEE isolates, and indicated that CEE and LI are related varieties of Eurasian TBE flavivirus that also includes TSE and RSSE strains.

Key words: Flavivirus; tick-borne encephalitis virus; louping-ill virus; antigenic similarity; monoclonal antibodies; immunofluorescence assay; taxonomy; nomenclature

Introduction

CEE and LI viruses belong to the TBE antigenic complex of the genus *Flavivirus* (family *Flaviviridae*) together with RSSE, Omsk haemorrhagic fever (OHF), Kyasanur Forest disease (KFD), LGT, Negishi (NEG), Powassan (POW), Karshi, Royal Farm, Carey Island and Phnom Penh bat viruses (Calisher *et al.*, 1989). The latter four viruses are not transmitted by ixodid ticks and are only distantly related to other viruses of the complex. On the other hand, LI, CEE and RSSE viruses are antigenically very closely related and there have always been problems with their differentiation (the use of non-conventional serological techniques is necessary), taxonomy and nomenclature (Casals and Webster, 1944; Clarke, 1962, 1964; Thorburn and Williams, 1966; Rubin and Chumakov, 1980; Reid, 1987; Calisher, 1988; Stephenson *et al.*, 1984, 1989; Shamanin *et al.*, 1990; Kopecký *et al.*, 1991b; Niedrig *et al.*, 1994). Our study was carried out to compare representative CEE and LI isolates obtained from diverse sources (ixodid ticks, mammals, birds) and countries, using MoAbs raised against the prototype LI virus, in a binding assay (IIFT).

Materials and Methods

Viruses. Twenty isolates of CEE virus (including the topotype Hypr; the prototype Stillerová was not available), 20 isolates of LI virus (including the prototype LI-31) and one TBE complex isolate from Turkey (TSE virus, strain TTE-80) were used in this study (Table 1). Two other Asian TBE complex members, RSSE (prototype Sofyin) and LGT (prototype TP-21) viruses were included as an outgroup for comparative purpose. The virus stocks were prepared as 10% (w/v) suspensions of infected suckling SPF mouse brains, clarified by centrifugation, and stored at –70 °C.

MoAbs against the prototype LI-31 virus were prepared and characterized previously (Hussain, 1990). Ascitic fluids were produced in 12-17 week-old BALB/c mice primed with tetramethyl pentadecane (Sigma) and injected intraperitoneally (ip) with cloned hybridoma cells (2 x 10^6) two weeks later. The ascitic fluid was clarified at 1000 x g and stored in 0.2 ml aliquots at –20 °C. Antibody isotype (immunoglobulin class and subclass) of individual

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Abbreviations: CEE = Central European encephalitis; FBS = foetal bovine serum; IIFT = indirect immunofluorescence test; ip = intraperitoneal(ly); KFD = Kyasanur Forest disease; LGT = Langat; LI = louping-ill; MoAb = monoclonal antibody; NEG = Negishi; OHF = Omsk haemorrhagic fever; PBS = phosphate buffered saline; POW = Powassan; RSSE = Russian spring-summer encephalitis; TBE = tick-borne encephalitis; TSE = Turkish sheep encephalitis

Table 1. Virus isolates used

Isolate	Source	Country	Locality	Year	Passage[1]	Isolated by
CEE virus isolates						
Hypr	patient (blood)	Moravia	Brno	1953	70	L.Pospíšil
Hypr/HL	patient (blood)	Moravia	Brno	1953	11[2]	L.Pospíšil
Očenáš	patient (blood)	Slovakia	Bratislava	1953	73	V.Bárdoš
Adamčík	patient (blood)	Slovakia	Bratislava	1968	4	M.Grešíková
A-58	bank vole (CNS)	Austria	Graz	1977	3	Z.Hubálek
Z-92C	bank vole (CNS)	Slovakia	Záhor.Ves	1990	4	O.Kožuch
Z-209	wood mouse (CNS)	Slovakia	Záhor.Ves	1990	4	O.Kožuch
Z-142C	yellow-necked m.	Slovakia	Záhor.Ves	1990	4	O.Kožuch
J-13	hedgehog	Slovakia	Nitra	1964	16	O.Kožuch
ZN-402	*Ixodes ricinus*	Moravia	Bítov	1976	2	Z.Hubálek
AI-123	*Ixodes ricinus*	Moravia	Cvilín	1984	4	J.Januška
AI-125	*Ixodes ricinus*	Moravia	Cvilín	1984	4	J.Januška
AI-272	*Ixodes ricinus*	Bohemia	Příbram	1986	6	J.Januška
K-470	*Ixodes ricinus*	Bohemia	Poteplí	1966	x	J.Kolman
CB-62	*Ixodes ricinus*	Bohemia	Kam.Újezd	1986	3	K.Křivanec
CB-117	*Ixodes ricinus*	Bohemia	Kaplice	1986	3	K.Křivanec
CB-263	*Ixodes ricinus*	Bohemia	Temelín	1987	4	K.Křivanec
D-269	*Ixodes ricinus*	Slovakia	Devín	1979	x	M.Grešíková
IR-155	*Ixodes ricinus*	Slovakia	Kurinec	1982	3	M.Grešíková
A-104	*Ixodes ricinus*	Austria	Graz	1990	3	O.Kožuch
LI and related virus isolates						
LI-31	sheep (brain)	Scotland	Edinburgh	1929	6	W.A.Pool
SB-526	sheep	Scotland	Oban	1968	3	J.Boyce
1131	sheep	Scotland	Inverness	1988	2	H.W.Reid
LI-G	pig	Scotland	Mull	1979	2	H.W.Reid
LI-K	grouse	Scotland	Grampian	1980	2	H.W.Reid
LI-I	sheep	Wales		1980	2	H.W.Reid
LI-A	sheep	England	Devon	1980	2	H.W.Reid
917	sheep	England	Penrith	1985	2	H.W.Reid
1065	sheep	England	Bristol	1985	2	H.W.Reid
1066	sheep	England	Bristol	1985	2	H.W.Reid
261	sheep	England	Newcastle	1987	2	H.W.Reid
2995	grouse	England	Thirsk	1987	2	H.W.Reid
2996	grouse	England	Thirsk	1987	2	H.W.Reid
MA-14	sheep	Ireland		1967	3	P.Lenihan
MA-27	cattle	Ireland		1968	3	P.Lenihan
MA-54	cattle	Ireland		1968	3	P.Lenihan
MR-46	*Ixodes ricinus*	Ireland		1971	3	P.Lenihan
2617	sheep	Spain	Basque	1987	2	H.W.Reid
2618	sheep	Spain	Basque	1987	2	H.W.Reid
Norway	sheep	Norway		1984	3	J.Krogsrud
TTE-80	sheep (brain)	Turkey	Gebze	1969	x	W.Hartley
RSSE virus						
Sofyin	patient (CNS)	Russia	Khabarovsk	1937	x	L.A.Zilber
LGT virus						
TP-21	*Ixodes granulatus*	Malaya	Kuala Lumpur	1956	x	C.E.G.Smith

[1]Number of suckling mouse brain passages (x, unknown).
[2]55 additional passages in HeLa cells.

MoAbs was determined by the double gel immunodiffusion test against specific anti-mouse Ig rabbit sera (Miles).

IIFT. Plastic tubes with flying glass coverslips were seeded with 2 x 10^5 pig kidney (IB/RS_2 clone 60) cells in medium 199 (Gibco) with 10% (v/v) foetal bovine serum (FBS), 10% (v/v) tryptose phosphate broth (Gibco) and antibiotics. Confluent monolayers were produced after 2 days at 37 °C; the growth medium was then aspirated, the cells were infected with 0.2 ml of virus suspensions containing about 10^4 $TCID_{50}$, and maintenance medium with 5% FBS was added 1 hr later. After an incubation at 37 °C for 40 hrs, the coverslips with cells were washed twice with phosphate buffered saline (PBS), dried, fixed with cold acetone for 10 mins and placed on microscope slides using DPX mountant (BDH). To block non-specific binding, a drop of 1:20 dilution of normal horse serum in PBS was added to each coverslip. After 30 mins incubation at 37 °C the coverslips were washed twice (2x10 mins) with PBS. One drop of each ascitic fluid (MoAb) diluted 1:50 to 1:200 in PBS was added to the coverslips. After 30 mins incubation at 37 °C the coverslips were washed with PBS twice as before. Then a 1:40 dilution in PBS of sheep anti-mouse immunoglobulin conjugated with FITC (Scottish Antigen Production Unit) was added (37 °C, 30 mins). After washing the cells were mounted in buffered glycerol-saline and examined for fluorescence using Leitz-Ortholux UV microscope and magnifications 125x and 500x. LI-negative mouse serum and non-infected cells were used in each test as controls. Only clear diffuse or granular cytoplasmic fluorescence was considered positive.

Computations. IIFT results were evaluated using the program NTSYS (Rohlf, 1990): Dice coefficient of similarity which ranges among the best measures of binary data (Hubálek, 1982) and UPGMA cluster analysis (Sneath and Sokal, 1973) were selected.

Results

Reactivity of the 43 virus isolates with 17 MoAbs is shown in Table 2. There were nine distinct reactivity patterns (groups) A-I:

(A) LI-31, SB-526, 1131, LI-G, LI-K, 2995, 2996, 1065, 1066;
(B) LI-I, LI-A, 917;
(C) 261;
(D) MA-54; MA-14, MA-27, MR-46, Norway;
(E) 2617, 2618 (SSE viruses);
(F) TTE-80 (TSE virus);
(G) all 20 isolates of CEE virus (cf. Table 1);
(H) Sofyin (RSSE virus):
(I) TP-21 (LGT virus)

The group A is formed by all LI Scottish isolates plus four isolates from upland England. The groups B and C are composed of sheep isolates from England and Wales, while the group D consists of the isolates from Ireland and Norway, the group E of two Spanish isolates, and the pattern F was observed in only one (Turkish) isolate. Interestingly, all CEE isolates including the cell-adapted Hypr/HL isolate and CB-263/D3, a spontaneous temperature-sensitive vari-

Table 2. Nine patterns of reactivity among the virus isolates against 17 MoAbs in IIFT

MoAbs	Ig[2]	Viruses[1]								
		LI-31 (9)	LI-I (3)	261 (1)	MA-54 (5)	SSE (2)	TSE (1)	CEE (20)	RSSE (1)	LGT (1)
		Pattern								
		A	B	C	D	E	F	G	H	I
LM 1.1	G1	+	+	+	+	+	-	-	-	-
LM 1.2	G1	+	+	+	+	+	-	+	+	-
LM 2.1	G1	+	+	+	+	+	+	+	+	-
LM 3.1	G1	+	+	-	+	+	+	+	+	-
LM 3.2	G1	+	+	+	+	+	+	+	+	-
LM 3.3	G1	+	+	+	+	+	+	+	-	-
LM 3.4	M	+	+	+	+	+	+	+	+	+
LM 4.1	G1	+	+	+	+	+	+	+	-	-
LM 4.2	G3	+	-	-	+	+	-	+	+	-
LM 7.1	G1	+	-	-	+	+	-	+	+	-
LM 7.2	A	+	+	-	+	-	-	-	-	-
LM 7.3	G1	+	+	+	+	+	+	+	+	-
LM 7.4	G2b	+	+	+	+	+	+	+	-	-
LM 7.6	G1	+	+	+	+	+	+	+	-	-
LM 8.1	G1	+	+	+	+	+	+	+	+	-
LM 8.2	G1	+	+	-	-	-	-	-	-	-
LM 9.2	M	+	+	+	+	+	+	+	+	+

[1]In parentheses, number of virus isolates with that reactivity pattern.
[2]Antibody isotype.

Table 3. Dice coefficient values (%) of antigenic relationships between the groups of isolates

	Virus groups[1]							
	A	B	C	D	E	F	G	H
A	100.0							
B	93.75	100.0						
C	82.76	88.89	100.0					
D	96.97	90.32	85.71	100.0				
E	93.75	86.67	88.89	96.77	100.0			
F	78.57	84.62	86.96	81.48	84.62	100.0		
G	90.32	82.76	84.62	93.33	96.55	88.00	100.0	
H	74.07	64.00	63.64	76.92	80.00	66.67	83.33	100.0
I	21.05	23.53	28.57	22.22	23.53	30.77	25.00	33.33

[1]The groups correspond to reactivity patterns in Table 2.

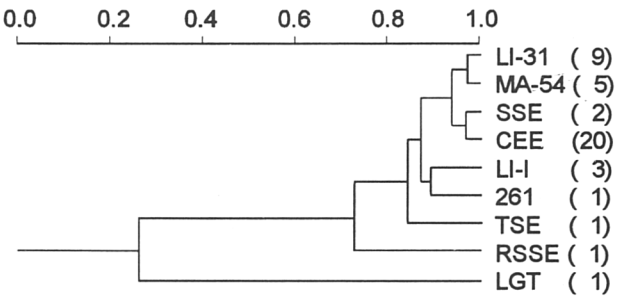

Fig. 1
Antigenic similarity among the flavivirus isolates based on IIFT, Dice coefficient (Table 3) and UPGMA cluster analysis
In parentheses, number of virus isolates of that reactivity pattern.

ant of CEE virus (Kopecký *et al.*, 1991a), reacted identically and formed group G. The MoAbs 4.2 and 7.1 are the only ones which had neutralizing and protective properties; MoAb 7.1 also revealed haemagglutinating ability (Hussain, 1990). The viruses non-reacting with these two MoAbs belong to the groups B, C and F.

Antigenic relationships of the viruses are summarized in the dendrogram (Fig. 1). The cophenetic correlation coefficient comparing the cluster analysis data with the original similarity matrix (Table 3) is highly significant ($r = 0.985$), and the dendrogram thus represents the matrix truly. Four clusters appear at the 85% similarity: groups A to G except F, and singletons F, H, and I. LGT virus is only distantly related to other viruses (26% similarity), while RSSE virus joins the European TBE viruses at the 72.7% overall similarity, and TSE virus does so at the 84.0% level. Within the first cluster, four subclusters appear: (a) the groups A and D joining at the 97.0% level; (b) SSE and CEE isolates (groups E, G) joining at the 96.6% level; (c) group B; (d) group C. While the subclusters (a) and (b) join at the 93.5% level, the subclusters (c) and (d) do so at the 88.9% level of antigenic similarity, and all subclusters (a) to (d) at 86.9%. The dendrogram demonstrates a much higher antigenic heterogeneity of LI isolates compared with CEE isolates. It is also clear from the data that LI virus could not be differentiated easily from CEE virus. E.g., the antigenic similarity between the prototype LI-31 and CEE isolates was higher (90.3%) than that between LI-31 and LI-261 (82.8%). The only MoAb differentiating CEE isolates from all LI isolates was LM 1.1, but this MoAb also reacted with SSE isolates that seem to be otherwise more closely related to CEE isolates (96.6% similarity) than to LI isolates (91.5% overall similarity).

Discussion

The IIFT revealed much greater antigenic heterogeneity among isolates of LI virus than among CEE isolates. The high antigenic homogeneity and stability of CEE isolates regardless of their source, year of isolation and passage history has already been stressed by several authors (Clarke, 1962; Heinz and Kunz, 1981, 1982; Heinz *et al.*, 1982, 1983; Guirakhoo *et al.*, 1987; Holzmann *et al.*, 1992; Whitby *et al.*, 1993a). From the biogeographic standpoint, the Eurasian TBE complex virus isolates circulating in areas close

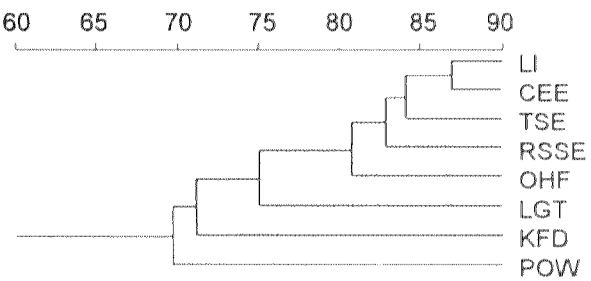

Fig. 2
Dendrogram showing the relationships of the TBE complex flaviviruses based on the nucleotide sequence homology of the E genes as published by Gao *et al.* (1993a) and Venugopal *et al.* (1994a)

to the limits of its geographic range are probably exposed to a number of ecological constraints that might result in their greater antigenic variability or evolutionary selection of 'antibody escape variants' (Holzman *et al.*, 1989; Jiang *et al.*, 1993; Gao *et al.*, 1994). E.g., Jiang *et al.* (1993) derived several neutralization-resistant mutants from a single LI isolate (369/T2), using MoAb 4.2. These mutants did not react in IIFT with MoAbs 4.2 and 7.1 which were also used in our study. We detected four LI isolates that did not bind MoAbs 4.2 and 7.1: LI-I, LI-A, 917 and 261. It means that these neutralization-resistant mutants do occur spontaneously. Interestingly, they result from a single nucleotide change. There might be a general parallel in the evolution of marginal and vicariant flavivirus populations to the island biogeography: a new insular population usually originates from a few propagules subjected to random genetic sampling, and environmental differences vs. mainland force genetic divergence, selection, adaptation and speciation (MacArthur and Wilson, 1967). There have been documented certain phenotypic and genomic changes of TBE viruses following their experimental serial passage in either vertebrates (Pressman *et al.*, 1993; Drokin *et al.*, 1994) or ixodid ticks (Labuda *et al.*, 1994).

Appendix

Accumulated antigenic, cross-protecting, peptide mapping and nucleotide sequencing data of a number of the TBE complex virus isolates show only minor differences between LI, CEE and RSSE viruses (Heinz and Kunz, 1982; Heinz *et al.*, 1983; Stephenson *et al.*, 1984; Guirakhoo *et al.*, 1991; Shiu *et al.*, 1991; Holzmann *et al.*, 1992; Venugopal *et al.*, 1992, 1994b; Gao *et al.*, 1993a; Gritsun *et al.*, 1993; Tsekhanovskaya *et al.*, 1993; this paper) and indicate that they probably represent just one virus species that can be conveniently named Eurasian TBE virus or *Flavivirus ixodis* in latinized binomial nomenclature. These viruses might be regarded as two subspecies (subtypes): *F. ixodis occidentalis* (Western subtype, with the varieties LI and CEE) and *F. ixodis orientalis* (RSSE, Eastern subtype). A similar view (without the nomenclatoric remarks) was already expressed by Clarke (1964) and Shiu *et al.* (1991). However, the differences between these two subspecies (subtypes) and the varieties are by no means clear-cut; *F. ixodis* rather seems to occur as a continuum or a gradient of antigenically and genetically similar forms over its vast geographic range. E.g., the Turkish TTE-80 strain differs from LI isolates in nucleotide homology and has been regarded as a distinct member of TBE complex (Gao *et al.*, 1993a; Whitby *et al.*, 1993b). It might represent another subspecies (subtype), or a variety of *F.i.occidentalis*. Neutralization and haemagglutination-inhibition tests fail to distinguish between TSE and LI viruses (Gao *et al.*, 1993a). Similarly, Marin *et al.* (1995) recently found that two strains (2617, 2618) of SSE virus, also used in our study (group E), shared 95 – 96% homology with the E protein of LI and CEE viruses. Despite this great similarity, they concluded that SSE virus is a new member of the TBE serogroup. However, the comparison of the amino acid homology among flaviviruses typically involves only a small number of strains, and their results should not always be regarded as conclusive for virus taxonomy. E.g. only typical strains of LI virus have usually been sequenced (LI-31, SB-526, NOR), whereas less typical isolates (261, LI-I, LI-A, 917) have not been studied. Their inclusion, however, could change the resulting pattern considerably.

Gao *et al.* (1993b) confirmed by the nucleotide sequence comparison of the E genes that the Norwegian TBE complex isolate is indistinguishable from LI (96% to 97% homology), while less related to CEE (85% homology); this correlates well with our IIFT results. The Norwegian isolate is thus up to now the only continental isolate that could be regarded as the LI variety of *F.i.occidentalis*, allowing speculations about the geographic spread of LI (from Scandinavia to the British Isles, or vice versa ?) and its means (immature *Ixodes ricinus* on migratory birds ?).

Of the other members of the TBE complex, NEG virus seems to be identical with LI virus (Venugopal *et al.*, 1992; Gao *et al.*, 1993a). OHF is related to RSSE virus (Gao *et al.*, 1993a; Tsekhanovskaya *et al.*, 1993) and might represent another subspecies (*F.i.omskii*) of *F. ixodis*. On the other hand, LGT, POW and KFD viruses are distantly related (Gao *et al.*, 1993a; Gritsun *et al.*, 1993; Mandl *et al.*, 1993; Tsekhanovskaya *et al.*, 1993; Venugopal *et al.*, 1994a,b) and represent separate species: *Flavivirus langat, F. powassani* and *F. kjasanurus*, respectively.

The envelope glycoprotein (E protein) of TBE complex viruses, a major structural protein carrying important biological functions (haemagglutinin, neutralization and protective immunity determinants), is highly conserved and

therefore evolutionary significant (Heinz and Kunz, 1982; Heinz *et al.*, 1983; Gao *et al.*, 1993a). By inspecting the dendrogram (Fig. 2) constructed by UPGMA clustering of the published data on nucleotide sequences of the E gene coding this protein (Guirakhoo *et al.*, 1991; Venugopal *et al.*, 1992, 1994a; Gao *et al.*, 1993a,b, 1994; Gritsun *et al.*, 1993; Mandl *et al.*, 1993), it seems that the nucleotide homology of about 80% to 82% (and 90% in the deduced amino acid identities of E protein) might indicate approximately the species level within the TBE complex flaviviruses. Corresponding homologies for the subspecies level could be approximately 85% and 93%, respectively. For a further clarification of the TBE complex taxonomy, it would be necessary to compare nucleotide sequences not only of the E gene but also of the genes coding other structural and nonstructural proteins in many representative isolates of the TBE complex, including those viruses not registered in the International Catalogue of Arboviruses but regarded as distinct serotype – e.g., Aina of Eastern Siberia (Rubin and Chumakov, 1980). In conclusion, the TBE antigenic complex (a subgenus: Calisher, 1988) of the genus *Flavivirus* seems to involve four ixodid-borne viral species, viz. *Flavivirus ixodis* (with a number of subspecies), *F. langat, F. kjasanurus, F. powassani.*

Note of the Editor-in-Chief

The authors are aware of the fact that the proposed nomenclature of viruses of concern used in Appendix was not yet accepted by the International Committee on Taxonomy of Viruses (ICTV). Therefore the Appendix should be regarded as a contribution of the authors to the current discussion on changes in the presently valid ICTV nomenclature of viruses of concern.

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References

Calisher CH (1988): Antigenic classification and taxonomy of flaviviruses (Family *Flaviviridae*) emphasizing a universal system for the taxonomy of viruses causing tick-borne encephalitis. *Acta Virol.* **32**, 469–478.

Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE (1989): Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal sera. *J. Gen. Virol.* **70**, 37–43.

Casals J, Webster LT (1944): Relationship of the virus of louping-ill in sheep and the virus of Russian spring-summer encephalitis in man. *J. Exp. Med.* **79**, 45–63.

Clarke DH (1962): Antigenic relationships among viruses of the tick-borne encephalitis complex as studied by antibody absorption and agar gel precipitin techniques. In Libíková H (Ed.): *Biology of Viruses of the Tick-Borne Encephalitis Complex.* Academia, Prague pp. 67–75.

Clarke DH (1964): Further studies on antigenic relationship among the viruses of the group B tick-borne complex. *Bull. W.H.O.* **31**, 45–56.

Drokin DA, Zlobin VI, Karganova GG, Vikhoreva TV, Yakimenko VV, Dzhivanyan TI, Lashkevich VA (1994): Changed genomes of tick-borne encephalitis virus strains after passages in mice. *Vopr. Virusol.* **39**, 160–162 (in Russian).

Gao GF, Hussain MH, Reid HW, Gould EA (1993a): Classification of a new member of the TBE flavivirus subgroup by its immunological, pathogenetic and molecular characteristics: identification of a subgroup-specific pentapeptides. *Virus. Res.* **30**, 129–144.

Gao GF, Hussain MH, Reid HW, Gould EA (1994): Identification of naturally occurring monoclonal antibody escape variants of louping ill virus. *J. Gen. Virol.* **75**, 609–614.

Gao GF, Jiang HW, Hussain MH, Venugopal K, Gritsun TS, Reid HW, Gould EA (1993b): Sequencing and antigenic studies of a Norwegian virus isolated from encephalomyelitic sheep confirm the existence of louping ill virus outside Great Britain and Ireland. *J. Gen. Virol.* **74**, 109–114.

Gritsun TS, Lashkevitsch VA, Gould EA (1993): Nucleotide and deduced amino acid sequence of the envelope glycoprotein of Omsk haemorrhagic fever virus; comparison with other flaviviruses. *J. Gen. Virol.* **74**, 287–291.

Guirakhoo F, Heinz FX, Mandl CW, Holzmann H, Kunz C, Grešíková M (1991): The relationship between the flaviviruses Skalica and Langat as revealed by monoclonal antibodies, peptide mapping and RNA sequence analysis. *J. Gen. Virol.* **72**, 333–338.

Guirakhoo F, Radda AC, Heinz FX, Kunz C (1987): Evidence for antigenic stability of tick-borne encephalitis virus by the analysis of natural isolates. *J. Gen. Virol.* **68**, 859–864.

Heinz FX, Berger R, Majdic O, Knapp W, Kunz C (1982): Monoclonal antibodies to the structural glycoprotein of tick-borne encephalitis virus. *Infect. Immun.* **37**, 869–874.

Heinz FX, Berger R, Tuma W, Kunz C (1983): A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. *Virology* **126**, 525–537.

Heinz FX, Kunz C (1981): Homogeneity of the structural glycoprotein from European isolates of tick-borne encephalitis virus: comparison with other flaviviruses. *J. Gen. Virol.* **57**, 263–274.

Heinz FX, Kunz C (1982): Molecular epidemiology of tick-borne encephalitis virus: peptide mapping of large non-structural proteins of European isolates and comparison with other flaviviruses. *J. Gen. Virol.* **62**, 271–285.

Holzmann H, Mandl CW, Guirakhoo F, Heinz FX, Kunz C (1989): Characterization of antigenic variants of tick-borne encephalitis virus selected with neutralizing monoclonal antibodies. *J. Gen. Virol.* **70**, 219–222.

Holzmann H, Vorobyova C, Ladyzhenskaya IP, Ferenczi E, Kundi M, Kunz C, Heinz FX (1992): Molecular epidemiology of tick-borne encephalitis virus: cross-protection between European and Far Eastern subtypes. *Vaccine* **10**, 345–349.

Hubálek Z (1982): Coefficients of association and similarity, based on binary (presence-absence) data: an evaluation. *Biol. Rev. (Camb.)* **57**, 669–689.

Hussain MH (1990): *A Study of Louping-Ill Virus Using Monoclonal Antibodies.* Ph.D. thesis: University of Edinburgh, 228 pp.

Jiang WR, Lowe A, Higgs S, Reid H, Gould EA (1993): Single amino acid codon changes detected in louping ill virus antibody-resistant mutants with reduced neurovirulence. *J. Gen. Virol.* **74**, 931–935.

Kopecký J, Křivanec K, Tomková E (1991a): Attenuated temperature-sensitive mutants of tick-borne encephalitis (TBE) virus isolated from natural focus. In Dusbábek F, Bukva V (Eds): *Modern Acarology.* Vol. 2. Academia and SPB Academic Publishing, Prague-The Hague, pp. 11–19.

Kopecký J, Tomková E, Grubhoffer L, Melnikova YE (1991b): Monoclonal antibodies to tick-borne encephalitis (TBE) virus: their use for differentiation of the TBE complex viruses. *Acta Virol.* **35**, 365–372.

Labuda M, Jiang WR, Kaluzová M, Kožuch O, Nuttall PA, Weisman P, Elečková E, Žuffová E, Gould S (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.

MacArthur RH, Wilson EO (1967): *The Theory of Island Biogeography.* The University Press, Princeton, 203 pp.

Mandl CW, Holzman H, Kunz C, Heinz FX (1993): Complete genomic sequence of Powassan virus-evaluation of genetic elements in tick-borne versus mosquito-borne flaviviruses. *Virology* **194**, 173–184.

Marin MS, McKenzie J, Gao GF, Reid HW, Antoniadis A, Gould EA (1995): The virus causing encephalomyelitis in sheep in Spain: a new member of the tick-borne encephalitis group. *Res. Vet. Sci.* **58**, 11–13.

Niedrig M, Klockmann U, Lang W, Roeder J, Burk S, Modrow S, Pauli G (1994): Monoclonal antibodies directed against tick-borne encephalitis virus with neutralizing activity in vivo. *Acta Virol.* **38**, 141–149.

Pressman EK, Malenko GV, Pogodina VV (1993): Variabilities in the antigenic structure of persisting tick-borne encephalitis virus strains. *Virus Res.* **30**, 295–301.

Reid HW (1987): Louping-ill. In Monath TP (Ed.): *The Arboviruses: Epidemiology and Ecology.* CRC Press, Boca Raton, pp. 117–134.

Rohlf FJ (1990): *NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System (Version 1.60).* Exeter Software, Setauket.

Rubin SG, Chumakov MP (1980): New data on the antigenic types of tick-borne encephalitis (TBE) virus. *Zentralbl. Bakt., Suppl.* **9**, 231–236.

Shamanin VA, Pletnev AG, Rubin SG, Zlobin VI (1990): Differentiation of strains of tick-borne encephalitis virus by means of RNA-DNA hybridization. *J. Gen. Virol.* **71**, 1505–1515.

Shiu SYW, Ayres MD, Gould EA (1991): Genomic sequence of the structural proteins of louping ill virus: comparative analysis with tick-borne encephalitis virus. *Virology* **180**, 411–415.

Sneath PHA, Sokal RR (1973): *Numerical Taxonomy.* W.H. Freeman, San Francisco, 573 pp.

Stephenson JR (1989): Classification of tick-borne flaviviruses. *Acta Virol.* **33**, 494.

Stephenson JR, Lee JM, Wilton-Smith PD (1984): Antigenic variation among members of the tick-borne encephalitis complex. *J. Gen. Virol.* **65**, 81–89.

Thorburn H, Williams H (1966): A serological examination of Scottish strains of louping-ill and their relation to other members of the complex. *Arch. Ges. Virusforsch.* **19**, 155–160.

Tsekhanovskaya NA, Matveev LE, Rubin SG, Karavanov AS, Pressman EK (1993): Epitope analysis of tick-borne encephalitis (TBE) complex viruses using monoclonal antibodies to envelope glycoprotein of TBE virus (*persulcatus* subtype): *Virus Res.* **30**, 1–16.

Venugopal K, Buckley A, Reid HW, Gould EA (1992): Nucleotide sequence of the envelope glycoprotein of Negishi virus shows very close homology to louping ill virus. *Virology* **190**, 515–521.

Venugopal K, Gritsun T, Lashkevich VA, Gould EA (1994a): Analysis of the structural protein gene sequence shows Kyasanur Forest disease virus as a distinct member in the tick-borne encephalitis virus serocomplex. *J. Gen. Virol.* **75**, 227–232.

Venugopal K, Reid HW, Gould EA (1994b): Tick-borne flavivirus NS1 gene: identification of conserved peptides and antigenic analysis of recombinant louping ill virus NS1 protein. *Virus Res.* **31**, 245–254.

Whitby JE, Jennings AD, Barrett ADT (1993a): Nucleotide sequence of the envelope protein gene of the tick-borne flavivirus, Kumlinge A52. *Virus Genes* **7**, 145–149.

Whitby JE, Whitby SN, Jennings AD, Stephenson JR, Barrett ADT (1993b): Nucleotide sequence of the envelope protein of a Turkish isolate of tick-borne encephalitis (TBE) virus is distinct from other viruses of the TBE complex. *J. Gen. Virol.* **74**, 921–924.