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Review

Epidemiology of Tick-Borne Encephalitis (TBE) and Phylogenetic Analysis of TBE Viruses in Japan and Far Eastern Russia

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CONTENTS:

SUMMARY: In Oshima, the southern part of Hokkaido, a tick-borne encephalitis (TBE) patient was found in 1993; in addition TBE virus was isolated from the blood samples of sentinel dogs, ticks pools, and rodents spleens in 1995 and 1996 by suckling mice. To identify when these TBE viruses emerged in Hokkaido, the times of divergence of TBE virus strains isolated in Oshima and Far Eastern Russia were estimated. TBE virus was isolated in Khabarovsk in 1998, and the nucleotide sequences of viral envelope protein genes of isolates from Oshima and Khabarovsk were compared. Based on the synonymous substitution rates of these virus E-protein genes, the lineage-divergence times of these TBE virus strains were predicted phylogenetically to be approximately 260-430 years ago. Furthermore, the virulence of TBE virus isolates from Oshima and Khabarovsk were compared in a mouse model. The results showed that the isolates possessed very similar virulence in mice. European TBE vaccine was found to be effective in TBE virus, Hokkaido strain. This review provides evidence that the Oshima strains of TBE virus in Hokkaido emerged from the Far Eastern Russia a few hundred years ago, which explains why the virulence of these strains is similar to that of TBE viruses isolated in Russia. Practical application of the vaccine should be considered in Japan.

Introduction

Tick-borne encephalitis (TBE) is a zoonotic arbovirus infection prevalent over a wide area on the Eurasian Continent including many European countries, Russia, Far Eastern Asia, and Japan (6,15). TBE virus causes severe encephalitis in humans with serious sequelae and has a significant impact on public health in these endemic regions.

TBE virus is a member of the family *Flaviviridae*, genus *Flavivirus*. The genus *Flavivirus* consists primarily of arboviruses and includes several important human pathogens endemic throughout the world such as dengue viruses (type 1 to 4), yellow fever virus, Japanese encephalitis (JE) virus, West Nile virus, and TBE viruses (Table 1) (2, 15, 26). On the basis of cross-neutralization tests and evolutionary tree analyses based on viral genome sequences, these viruses have been divided into several groups (Table 1). The flavivirus virions are 40-50 nm in diameter, spherical in shape, and contain a core and an envelope. The genome is a single positive-strand RNA (approximately 11-Kb in the length) encoding three structural proteins (capsid protein C, membrane precursor protein prM, and envelope protein E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) within a single long open reading frame. It is believed that the 5'- and 3'- termini of the genome (5'- and 3'-non-coding regions [NCR]) consist of a predicted RNA folding pattern and contain important elements for viral replication, translation, and packaging of the genome (3, 14). The viral genome RNA is infectious and gives rise to the production of viral particles when introduced into susceptible cells.

TBE was first described by an Austrian physician in 1931 (21). In 1937, a virus was isolated from the brain of an encephalitis patient in the southern far-east region by former Soviet scientist Zilber (28). It was shown that the virus is transmitted to humans by tick bites (17). The main reservoirs of TBE virus are small rodents, with ticks acting as a vector. Although several species of ticks can be infected with TBE

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Table 1. List of major flavivirus groups and viruses

Group	Viruses	Vector	Geographic distribution
Tick-borne encephalitis (TBE) complex	Russian spring-summer encephalitis [Far Eastern subtype TBE]	Tick	Far Eastern Russia and Asia, Japan
	Central European encephalitis [European subtype TBE]	Tick	Central and Eastern Europe
	Louping ill	Tick	British Isles, Ireland
	Langat	Tick	Asia
	Omsk hemorrhagic fever	Tick	Asia
	Kyasanur Forest disease	Tick	Asia
	Powassan	Tick	North America, Russia
Japanese encephalitis complex	Japanese encephalitis	Mosquito	Far Eastern Russia and Southeast Asia, Japan, Korea, China, India
	St. Louis encephalitis	Mosquito	America
	Murray Valley encephalitis	Mosquito	Australia, New Guinea
	Kunjin	Mosquito	Australia
	West Nile	Mosquito	Africa, Europe, Asia, America
Dengue	Dengue type 1	Mosquito	Tropical areas of Asia, Australia, Oceania, Africa, America
	Dengue type 2		
	Dengue type 3		
	Dengue type 4		
Yellow fever	Yellow fever	Mosquito	Africa, South America
No known vector	Rio Bravo	Unknown	America
	Apoi	Unknown	Japan

virus, *Ixodes persulcatus* and *I. ricinus* account for essential transmissions to humans. TBE virus can chronically infect ticks throughout their lives and can be transmitted transovarially and sexually (6,10). Human infections mainly occur by tick bite in the spring and early summer, corresponding to periods of tick activity. On the other hand, humans can also be infected by raw milk or cheese from TBE virus-infected goats, sheep, or cows.

TBE virus has been subdivided into two subtypes; the Far Eastern subtype known as Russian spring-summer encephalitis (RSSE) virus, and the Western European subtype known as Central European encephalitis (CEE) virus. Their main vector ticks are different; the Far Eastern subtype is transmitted by *I. persulcatus* and the Western European subtype by *I. ricinus*. They also show different clinical manifestations and mortality rates in human. The Far Eastern subtype disease is regarded as more severe and prolonged than the Western European subtype disease. The incubation period of TBE is usually 7-14 days. In the Far Eastern subtype disease, cases show a monophasic course. Onset is generally abrupt, with fever, headache, flushing of the face and neck, conjunctival injection, somnolence, nausea, vomiting, dizziness, and myalgia. The following are the encephalitic syndromes with severe pain in the arms and legs, back, hyperesthesia, asymmetrical paresis of cranial nerves, tremor, ataxia, sensory disturbances, and unconsciousness. Permanent paresis occurs in 5-30% of cases, and the case-fatality rate is 5-20%. On the other hand, in the Western European subtype disease, cases show a biphasic course and there are no signs or symptoms of meningoencephalitis in the first phase. In approximately one-third of patients, the second phase of the disease develops with signs and symptoms of meningoencephalitis (6). Permanent paresis occurs in 2-10% of cases, and the case-fatality rate is 0.5-2.0%.

In the 1990s, total annual TBE case numbers were 6,000-10,000, and most cases were from Russia. In Russia, TBE case numbers in recent years have been increasing, with 5,486 cases in 1990, 7,893 cases in 1993, and 9,548 cases in 1996. Such a drastic increase occurred because many city dwellers came into contact with ticks during visits to gardens or dachas located near big cities. TBE cases have also occurred in rural areas in the 1950s and 1960s (10).

There is no curative therapy for TBE. In Russia, specific immunogloblin injection is used to protect against outcomes of TBE disease. Vaccination appears to be the most effective means of preventing TBE. Currently, several vaccines are used in Europe and Russia. The most widely used vaccine in Europe is FSME-Immune Inject (Immune AG, Vienna, Austria), which consists of purified and formaldehyde-inactivated whole virus. In Austria, mass vaccination and active surveillance resulted in a dramatic decline in the incidence of TBE virus infection (9,11-13).

In Japan, no confirmed TBE cases had been reported for many years. However, in 1993 in Oshima district, Hokkaido, a severe encephalitis case with neurological symptoms and convulsions was diagnosed as viral encephalitis based on cerebrospinal fluid examinations at acute and convalescent phases of the patient. We describe the epidemiology of this case and TBE virus isolation from sentinel dogs in Hokkaido, Japan. To obtain the preventive measures for TBE virus in Japan, we evaluated European TBE vaccine to TBE virus, Hokkaido strain. Because of their geographical closeness, it was considered that the ancestor virus of Hokkaido strain had been diverged from far eastern region of Russia, where TBE is prevalent. Therefore, the divergence time of TBE viruses from Hokkaido and Far Eastern Russia was estimated. Furthermore,

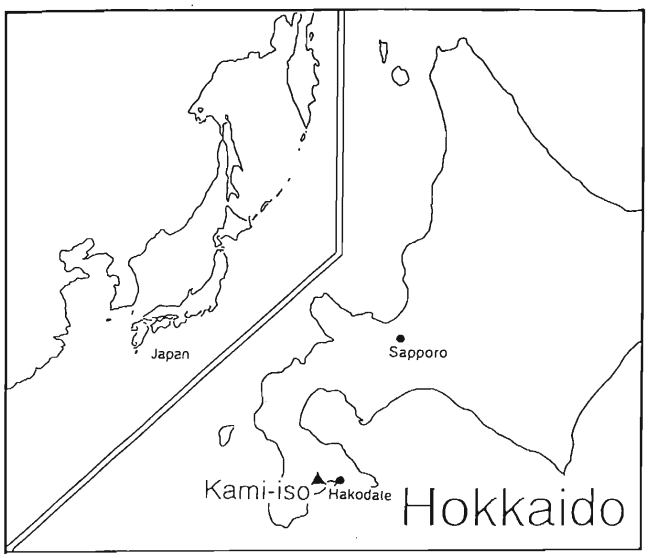

Fig. 1. Geographical location of a case-study area, Kamiiso (▲).

the virulence of TBE virus strains recently isolated from various hosts in Hokkaido and Far Eastern Russia were also compared.

Epidemiology of TBE and isolation of TBE virus in Hokkaido, Japan

1. Clinical findings and virus isolation

In the town of Kamiiso, Oshima, Japan, a dairy farmer's wife (37 years old) suddenly suffered a febrile illness with a temperature of 39°C on October, 1993. She was hospitalized on the 3rd day after the onset of illness. Double vision appeared on the 2nd hospital day, convulsions on the 5th day. She was under artificial ventilation from days 5 to 20. Remaining motor paralysis of the arms and neck was present 3 years later. Cerebrospinal fluid was tested at the acute and convalescent phases, and clinical data for the respective phases showed cell counts of 468 and 6 cells/mm^3, and glucose concentrations 76 and 69 mg/dl (25). The results showed that she suffered from encephalitis or encephalomyelitis of viral origin.

The geographical location of the case-study area is shown (Fig. 1). There were four farms within a 1.0-km radius. The area was deforested 50 years ago. The sera and cerebrospinal fluid were examined by ELISA for JE virus (Table 2). The ELISA IgG titer for JE virus increased significantly from the acute phase to the convalescent phase. However, the ELISA IgM titer essential to JE diagnosis was negative. Therefore, another flavivirus infection was suspected as the cause of encephalitis in this case, and a more specific neutralization test (NT) was performed including JE virus strain JaGAr-01, Negishi virus isolated in 1948 in Japan (1), Apoi virus isolated from rodents in Hokkaido in 1954 (20), and RSSE virus strain Sofjin. NT antibody titers increased significantly to RSSE virus at acute and convalescent phases among flaviviruses tested (16). The results indicated that the patient was infected with a TBE-related virus.

In 1995, we kept 10 sentinel dogs in the area in a free-living environment and collected serum samples once a week from April to July (22). NT titers of the dog sera to Langat virus were determined, and virus isolation was carried out (Table 3). On April 22, all 10 dogs had a negative antibody to Langat virus. Two animals converted to positive on May 4th, three on May 13th, and five on May 27th. The results clearly showed that the virus was prevalent in the area in 1995. The blood was inoculated into suckling mice via an intracerebral route. Three viral strains were isolated from the blood of sentinel dogs No. 5, 8, and 9, all of which were obtained 7 days prior to the sero-converted day. The virus strains from dogs No. 5, 8, and 9 were designated as Oshima-3-6, -5-10, and -5-11, respectively. The antigenicities of the virus isolates were examined by indirect immunofluorescent-antibody assay (IFA) test using monoclonal antibodies supplied by Dr. F. X. Heinz at the University of Vienna, Austria (22). Three of the isolates showed high titers to TBE-complex-specific 2E7 and TBE-type-specific 7G7 monoclonal antibodies (Table 4). The

Table 3. Monitoring virus activity and virus isolation using sentinel dogs in 1995

Dog No.	NT[1] antibody titer to TBE (Langat) virus				
	April 22	May 4	May 13	May 20	May 27
1	<20	<20	<20	<20	<20
2	<20	<20	<20	<20	<20
3	<20	<20	<20	<20	<20
4	<20	160	80	40	80
5	<20	<20*	640	320	160
6	<20	80	80	80	80
7	<20	<20	<20	<20	<20
8	<20	<20	<20	<20*	160
9	<20	<20	<20	<20*	160
10	<20	<20	<20	<20	<20

[1] Neutralization test (NT).
*; Viruses were isolated from blood of sentinel dogs by suckling mouse inoculation. (ref. 22)

Table 2. Serological examination on sera and cerebrospinal fluid of the patient

Sample (days)	Anti-JE[1] antibody		Anti-flavi NT[2] antibody			
	IgG-ELISA	IgM-ELISA	JE	RSSE[3]	Negishi	Apoi
Serum (6 days)	1,600	<100	10	640	40	10
Serum (43 days)	16,000	<100	20	2,560	320	10
CSF[4] (52 days)	1,600	<10	nt[5]	nt	nt	nt

[1] Japanese encephalitis (JE). (ref. 16)
[2] Neutralization test (NT).
[3] Russian spring-summer encephalitis (RSSE).
[4] Cerebrospinal fluid (CSF).
[5] Not tested (nt).

Table 4. Identification of virus isolates by IFA test using monoclonal antibodies

Specificities (MAb)[1]	IFA[2] titers to				
	JE[3]	Langat	Oshima-5-10	Oshima-5-11	Oshima-3-6
Group-reactive (6E2)	>6400	1600	>6400	>6400	>6400
TBE-complex-specific (2E7)	<100	>6400	>6400	>6400	>6400
Type-specific (7G7)	<100	<100	1600	1600	1600

[1] Monoclonal antibody (MAb). (ref. 22)
[2] Indirect immunofluorescent antibody assay (IFA).
[3] Japanese encephalitis (JE).

viral strains isolated were identified as TBE-related virus. In 1995 and 1996, TBE virus strains were isolated from *I. ovatus* ticks and rodents (23, 24).

2. Genetic characterization of the virus isolates

The E-protein gene sequence of one of the isolates, Oshima-5-10 strain, was determined. The whole E-protein gene was amplified by RT-PCR and directly sequenced. The sequence of Oshima virus was compared with those of TBE complex viruses (Table 5). The nucleotide identities of Oshima virus with RSSE (Far Eastern subtype) and Western TBE (WTBE) were 95.7% and 84.3%, respectively. Amino acid sequences of Oshima virus showed extremely high identities with those of RSSE (99.0%) and WTBE (95.8%).

Phylogenetic analysis of the E-protein gene was carried out to study the genetic relationship between Oshima virus and other TBE complex viruses (22). Oshima virus showed the closest relationship with RSSE virus among other TBE complex viruses by the unweighted pair group method with arithmetic means (UPGMA) method (Fig. 2) and a similar result was also obtained by the Neighbor-Joining method (results not shown). The high reliability of the tree was ascertained by Majority-rule and strict consensus tree program; RSSE versus Oshima was 1,000/1,000.

Phylogenetic analysis of TBE viruses from Japan and Far Eastern Russia

1. Relatedness of TBE virus, Hokkaido strain and Far Eastern Russian strains

To reveal the relationship among TBE viruses from Japan and Far Eastern Russia, we isolated TBE viruses from ticks collected in Khabarovsk, Russia in 1998 by the suckling mouse inoculation method and genetically analyzed these and Hokkaido isolates. We determined the nucleotide sequence of the 1,488 nucleotide long E gene from three strains (KH98-2, -5, and -10) isolated in Khabarovsk (8), five strains isolated in Hokkaido from dog blood (Oshima 5-11, 3-6), *I. ovatus* (Oshima I-1), *Apodemus speciosus* (Oshima A-1) and *Clethrionomys rufocanus* (Oshima C-1) and the low-passage Sofjin virus (Sofjin-HO) (22-24). The data of Sofjin-HO showed 37 nucleotide and two amino acid differences from the data of Sofjin virus strain determined by Pletnev et al. (18). Identities of the E-protein gene nucleotide sequences between the Khabarovsk isolates were 98.8-99.5%, between the Oshima isolates were 99.0-99.9%, and between the Khabarovsk isolates and the Oshima isolates were 95.2-95.7% (data not shown). All pairs among these virus isolates showed very high identities, and pairs of isolates from the same geographical area had extremely high identities. The position and variety of deduced amino acid changes among Oshima and Khabarovsk strains are shown in Fig. 3. Amino acid variations were found only at 14 different positions, which shows the high degree of conservation of amino acid sequences among these viruses. Differences in three of the 14 different positions

Table 5. Identities of the nucleotide and deduced amino acid sequences of Oshima virus with those of TBE complex viruses

Virus	Identity (%)	
	Nucleotide	Amino acid
*Russian spring-summer encephalitis	95.7	99.0
Western tick-borne encephalitis	84.3	95.8
Louping ill	81.9	91.3
Spanish sheep encephalitis	81.8	92.9
Turkish sheep encephalitis	81.8	93.5
Omsk hemorrhagic fever	80.3	94.0
Langat	75.3	88.7
Kyasanur Forest disease	72.2	81.2
Powassan	69.8	78.1

*Far Eastern tick-borne encephalitis. (ref. 22)

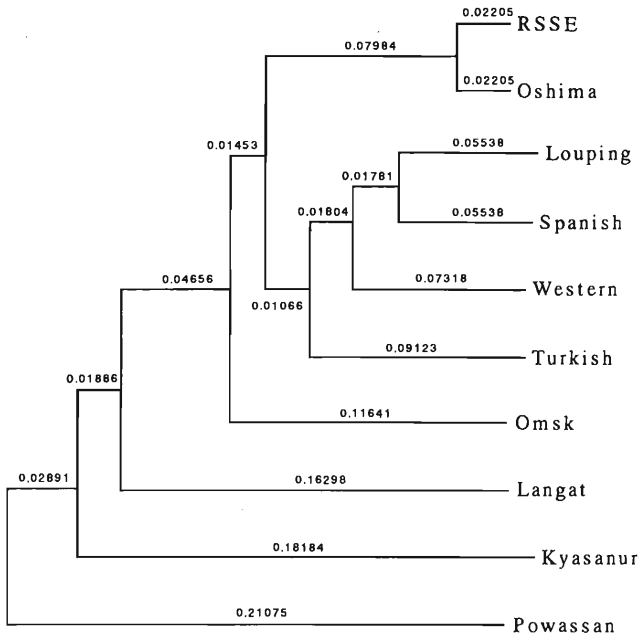

Fig. 2. Phylogenetic tree of TBE complex viruses. The nucleotide sequence of the E-protein gene of Oshima virus obtained in the study was compared with those of other TBE complex viruses available in DNA databases. The tree shown above was obtained by the UPGMA method. Virus strains used for the analysis are as follows; Russian spring summer encephalitis (RSSE), Oshima 5-10 (Oshima), louping ill (Louping), Spanish sheep encephalitis (Spanish), Western TBE (Western), Turkish sheep encephalitis (Turkish), Omsk hemorrhagic fever (Omsk), Langat (Langat), Kyasanur Forest disease (Kyasanur), and Powassan (Powassan). (ref. 22)

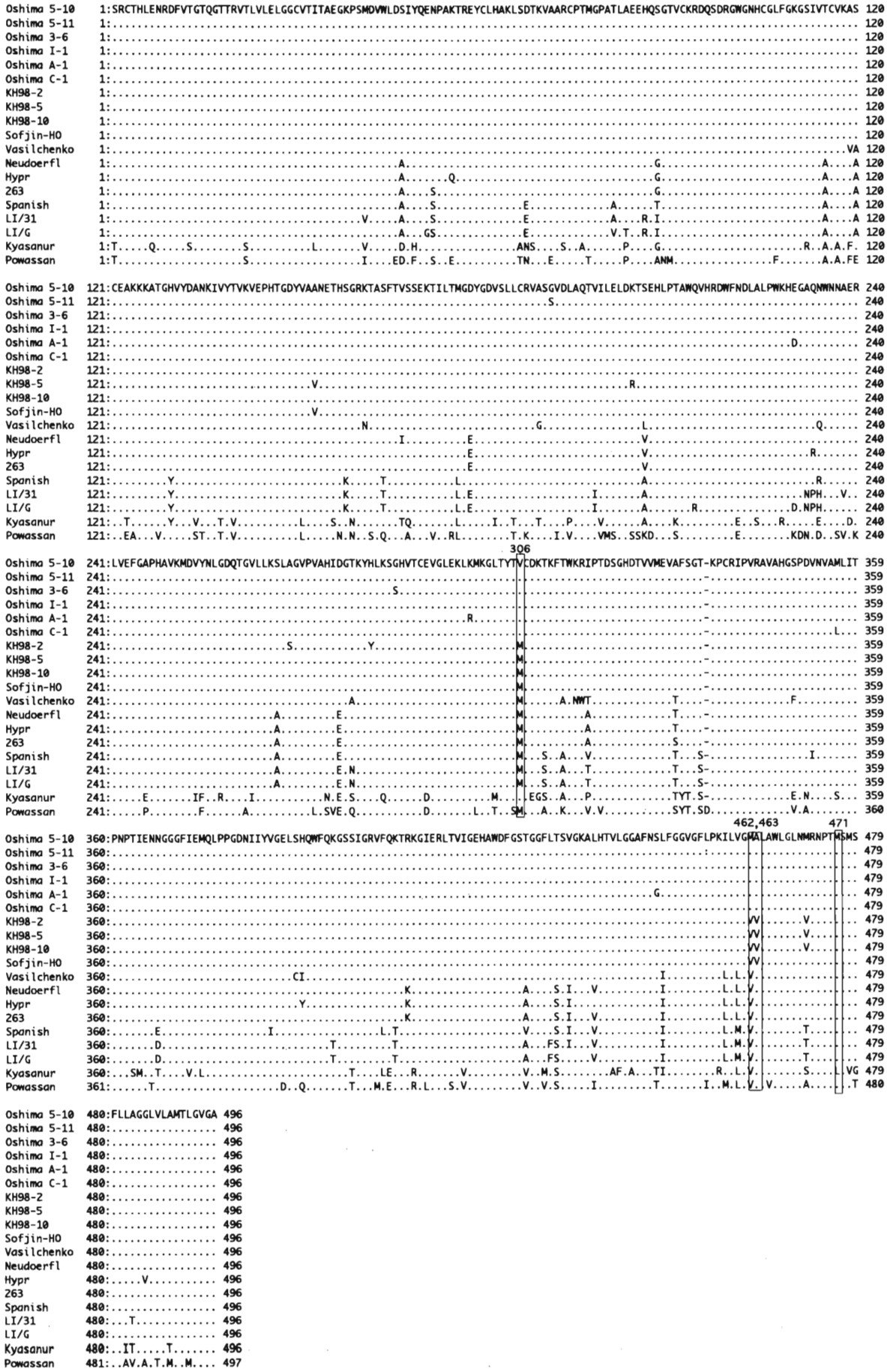

Fig. 3. Comparison of the amino acid sequences deduced from the E-gene sequences of various TBE complex viruses. LI, Louping ill virus. (ref. 8)

(positions 306, 462, and 463) were found only among Khabarovsk isolates, including Sofjin-HO. Other variations were found randomly, and a consistent distribution was not found. With reference to the three-dimensional structure of the E protein of TBE virus strain Neudoerfl (19), most amino acid changes were located on the upper or lateral surface (data not shown). No specific amino acid changes in the E protein were found in KH98-10, even though this strain showed a lower virulence than other strains.

Amino acid position 471 is valine in the Khabarovsk isolates, which is unique among TBE complex viruses, even when other sequenced flaviviruses are compared (Fig. 3). This implies that the Khabarovsk strains have evolved independently of the standard Far Eastern TBE virus strains and also independently of the Japanese isolates. Amino acid position 462 is alanine in the Hokkaido isolates but valine in the Khabarovsk and Sofjin viruses. Alanine is also found in Western European TBE viruses, louping ill virus and Kyasanur Forest disease (KFD) and Powassan (POW) viruses. This again implies independent evolution between the Japanese and Russian TBE viruses. Moreover, since the alanine residue is common to most of the other tick-borne flaviviruses, this implies that the Russian isolates have lost this genetic marker.

The amino acid alignment also revealed four amino acids unique to Western European TBE virus isolates, supporting the clinal concept of tick-borne virus evolution across Eurasia (27). Their respective positions are 88 (G), 206 (V), 317 (A), and 407 (K).

A phylogenetic tree constructed from synonymous distances of TBE serocomplex viruses is shown in Fig. 4. Virus strains isolated in Hokkaido and Khabarovsk, including Sofjin-HO virus, form a cluster with 100% bootstrap support. Thus, these virus strains were classified as Far Eastern subtype TBE virus, since strain Sofjin has been proposed as a prototype of the Far Eastern subtype (7). The branching pattern of the phylogenetic tree (Fig. 4) clearly distinguishes the Far Eastern subtype TBE complex viruses from the Siberian subtype Vasilchenko and also the European and UK TBE complex viruses. The tree also shows that two independent but closely related lineages have developed following introduction of the virus into Far Eastern Russia: the Khabarovsk lineages, including Sofjin virus, which became established in the far eastern part of Russia, and the Hokkaido lineage, consisting of the Oshima strains, which was subsequently introduced into Japan. In view

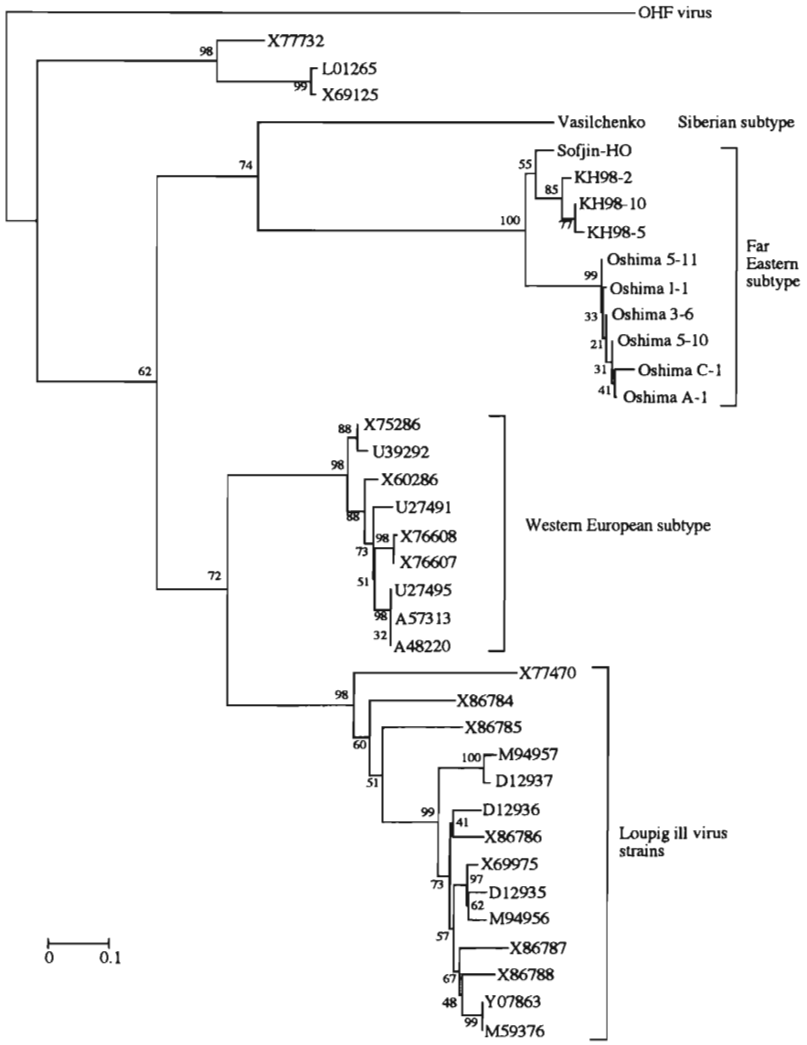

Fig. 4. Phylogenetic tree of TBE virus strains containing the Far Eastern subtype, Siberian subtype and Western subtype virus strains. The tree was rooted using 1488 nucleotides of viral E-gene sequences and Omsk haemorrhagic fever (OHF) virus as the outgroup virus. Virus strains are indicated as accession no. from DDBJ, EMBL, and Gene Bank nucleotide sequence databases. The scale bar indicates the number of substitutions per nucleotide site. (ref. 8)

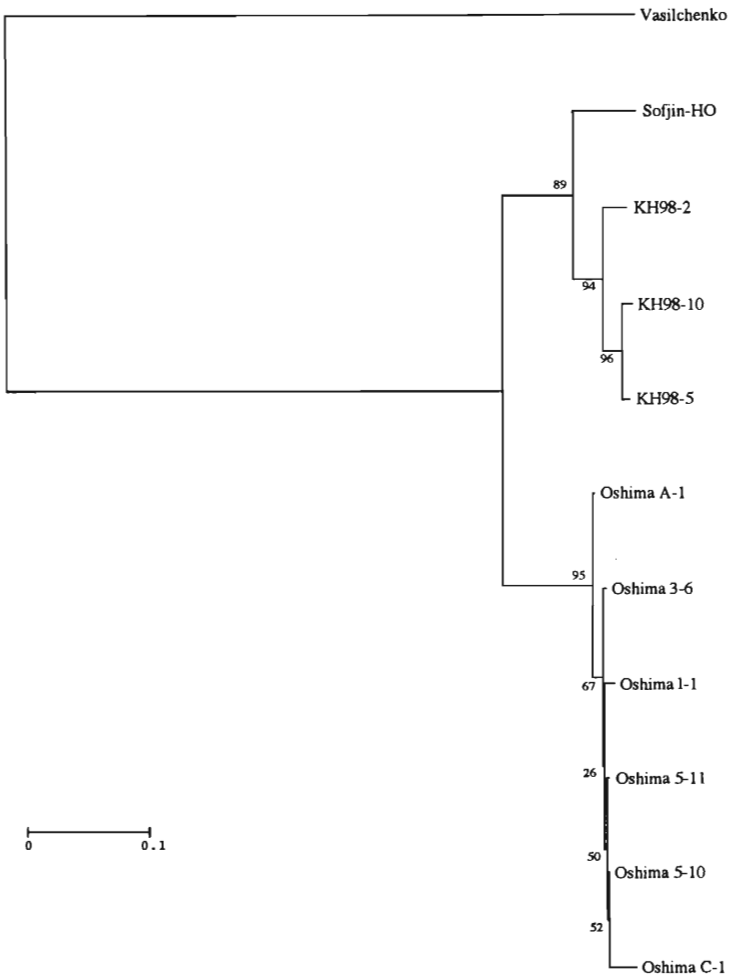

Fig. 5. Phylogenetic tree of the Far Eastern subtype TBE virus strains isolated in Oshima and Khabarovsk. (ref. 8)

of the Far Eastern subtype being distributed over a very wide geographical area of Russia and being distinguishable from the Siberian subtype TBE viruses such as Vasilchenko (7), it seems reasonable to assume that the new Oshima viruses dispersed in an eastward direction from Russia to Japan rather than from Japan to Russia.

2. Divergence of TBE virus, Hokkaido strain from Khabarovsk strains

A phylogenetic tree constructed from synonymous distances of KH98-2, -5, and -8, Sofjin-HO and Oshima strains and the Vasilchenko strain as an outgroup is shown in Fig. 5 (8). The tree shows that strains from Far Eastern Russia and strains from Hokkaido form distinct clusters with good bootstrap support, 89% and 95%, respectively. We estimated the synonymous substitution rates of these strains. The average synonymous substitution rate of these strains was estimated to be 2.9×10^{-4} per site per year. The time of divergence of the Khabarovsk and Hokkaido strains was calculated by using the average substitution rate and the synonymous distances shown in Fig. 5. The result showed that these virus strains had diverged approximately 260-430 years ago. Therefore, since the Hokkaido strains are genetically distinct from the Far Eastern subtype TBE viruses, they may have dispersed quite soon after diverging from the Far Eastern subtype viruses.

Pathogenicity of TBE virus, Hokkaido strain and protection from infection by European vaccine

1. Pathogenicity of the Hokkaido isolates

We compared the pathogenic characteristics among Hokkaido isolates and other strains of TBE virus (4). The degrees of neuroinvasiveness of the viruses were examined by subcutaneous (s.c.) inoculation into the axilla of mice. Mice were also inoculated intracerebrally (i.c.) to evaluate the degree of neurovirulence. In addition, intraperitoneal inoculation was employed to assess the neuroinvasiveness of the virus and to establish a mouse model for vaccine evaluation by determination of 50% lethal dose (LD_{50}) values.

The differences in neurovirulence and neuroinvasiveness among TBE virus, Oshima, Sofjin and Hochosterwitz strains, and TBE complex virus TP-21 strain are shown in Fig. 6, Fig. 7, and Table 6.

Mice were inoculated s.c. with 10,000 focus-forming units (FFU) of each strain (Fig. 6a) and significant differences in neuroinvasiveness were revealed among the strains. Seventy-five percent of the mice inoculated with TP-21 strain survived, but only 12.5, 0, and 0% of those inoculated with Oshima, Sofjin, and Hochosterwitz strains survived, respectively. Moreover, the survival times of mice inoculated with Sofjin and Hochosterwitz strains were significantly shorter than those with Oshima strain (mean survival times 9.5, 11.6, and 14.1 days, respectively, $P < 0.05$; Oshima versus Sofjin and

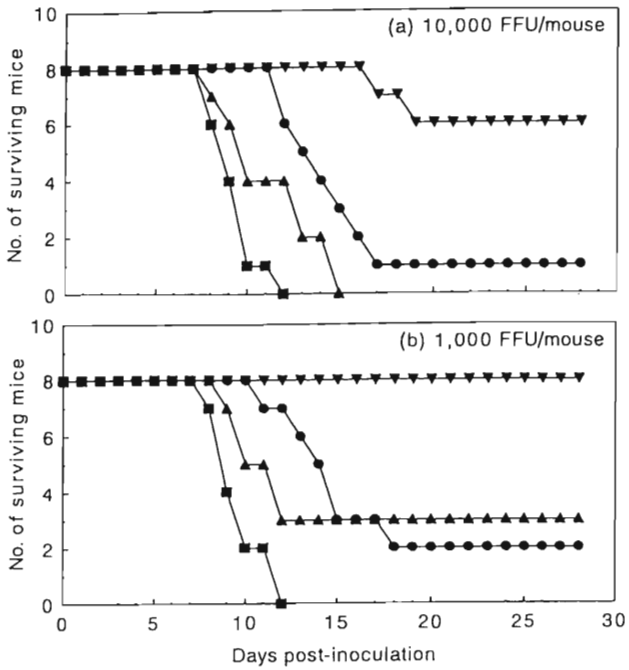

Fig. 6. Survival of adult mice subcutaneously inoculated with TBE virus strains. Mice were inoculated with 10,000 (a) or 1,000 FFU (b) of TBE virus strains Oshima 5-10 (●), Sofjin (■), Hochosterwitz (▲), or TP-21 (▼), respectively. Survival was recorded for 28 days post-inoculation. (ref. 4)

Oshima versus Hochosterwitz), however death from strain TP-21 was delayed (mean survival time 18.0 days). Similar results were obtained when 1,000 FFU was injected s.c. (Fig. 6b), whereas none of the mice were killed by TP-21 strain. When 10 FFU was injected s.c., only one mouse was killed by Sofjin strain, and no clear differences among TBE strains were observed (data not shown).

We examined the neurovirulence by intracerebral inoculation of 10 FFU, and the results are shown in Fig. 7. Sofjin strain was the most neurovirulent among the strains examined in this experiment (mean survival time 6.5 days, $P < 0.05$; Oshima versus Sofjin and Hochosterwitz versus Sofjin). As can be seen in Fig. 6, Oshima strain was significantly less pathogenic in its neuroinvasiveness than Hochosterwitz strain. Whereas, no significant difference was observed between the mean survival time of mice inoculated i.c. with Oshima strain

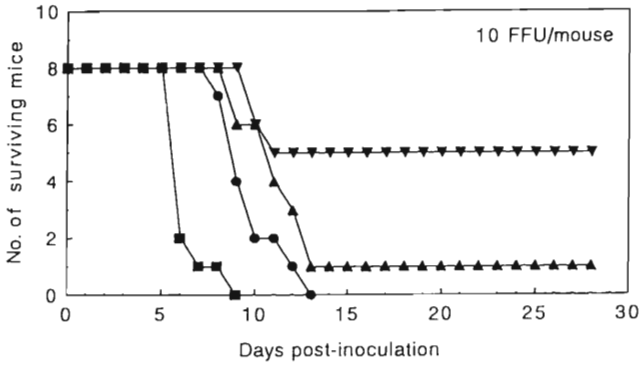

Fig. 7. Survival of adult mice intracerebrally inoculated with TBE virus strains. Mice were inoculated with 10 FFU of TBE virus strains Oshima 5-10 (●), Sofjin (■), Hochosterwitz (▲), or TP-21 (▼). Survival was recorded for 28 days post-inoculation. (ref. 4)

and that of mice inoculated i.c. with Hochosterwitz strain (10.0 versus 11.1 days). All or almost all mice were killed by Oshima, Sofjin, and Hochosterwitz strains, but 62.5 % of mice inoculated with TP-21 strain survived.

The mortality rates and mean survival days of mice inoculated intraperitoneally (i.p.) with Oshima, Hochosterwitz, or Sofjin strain are shown in Table 6. The LD_{50} of Oshima strain was approximately 10- to 20-fold higher than that of Hochosterwitz and Sofjin strains, and the mean survival time for each virus dilution of Oshima strain was significantly longer than that of the other two strains.

In conclusion, the degree of neuroinvasiveness of the strains s.c. inoculated was Sofjin = Hochosterwitz > Oshima > TP-21, and in mice inoculated i.p. was Sofjin = Hochosterwitz > Oshima. Neurovirulence, as determined after intracerebral inoculation, was Sofjin > Oshima = Hochosterwitz > TP-21.

Virus replication in the brains were compared in the s.c. and i.c. inoculated mice with Oshima and Sofjin strains (Fig. 8). In mice inoculated s.c. with Sofjin strain (10,000 FFU/mouse), the virus was first detected in the brain on day 5 (1.1×10^5 FFU/g) , and the titer increased progressively, reaching a maximum titer (1.5×10^9 FFU/g) on day 8 (Fig. 8a). In mice inoculated s.c. with Oshima strain (10,000 FFU/mouse), the virus was first detected in the brain on day 6 (5×10^2 FFU/g), and the titer increased gradually, reaching its maximum (3×10^5 FFU/g) on day 10. Of 7 mice inoculated with Sofjin strain, 4 individuals in which the virus titer exceeded $5.2 \times$

Table 6. Mortality of mice intraperitoneally inoculated with TBE viruses

Dose (FFU/mouse)	Oshima 5-10		Sofjin		Hochosterwitz	
	Mortality[1]	Survival time[2]	Mortality	Survival time	Mortality	Survival time
1,000	9/10	14.3±2.9[3,4]	10/10	8.2±0.8[3,5]	10/10	9.6±1.3[4,5]
100	6/10	13.3±2.4[3,4]	10/10	8.8±0.6[3]	9/10	9.1±1.2[4]
10	6/10	15.5±4.0[3,4]	10/10	9.6±0.7[3]	10/10	10.1±1.3[4]
1	4/10	15.5±2.4[3,4]	6/10	10.5±1.9[3]	9/10	10.6±1.5[4]
0.1	0/10		0/10		1/10	11.0
0.01	0/10		0/10		0/10	
LD_{50} (FFU/mouse)	≧ 8.13		0.68		0.36	

[1] number of deaths / total number tested. (ref. 4)
[2] Mean±SD (days).
[3] $P < 0.05$ Oshima vs Sofjin.
[4] $P < 0.05$ Oshima vs Hochosterwitz.
[5] $P < 0.05$ Sofjin vs Hochosterwitz at each dose.

10^8 FFU/g were dead, whereas only one mouse inoculated with Oshima strain, which had a virus titer of 1.2×10^6 FFU/g, was dead on day 10.

After mice were inoculated i.c. with Sofjin strain (10 FFU/mouse), the virus was first detected in the brain on day 2 (3.6×10^2 FFU/g); this titer represented an approximately 10-fold increase over the original inoculum (Fig. 8b). The titer increased similarly as in the case of s.c. inoculation, reaching a maximum (1.1×10^9 FFU/g) on day 6. In mice inoculated i.c. with Oshima strain (10 FFU/mouse), the virus was first detected in the brain on day 3 (2.2×10^3 FFU/g), and the titer increased gradually between 3 to 5 days post-inoculation (p.i.), followed by an abrupt increase between 5 to 6 days p.i. Of mice inoculated i.c. with Sofjin strain, some mice died during the observation period, and the virus titers in the brains of these mice exceeded 1.6×10^8 FFU/g. None of the mice inoculated i.c. with Oshima strain died during the experiment.

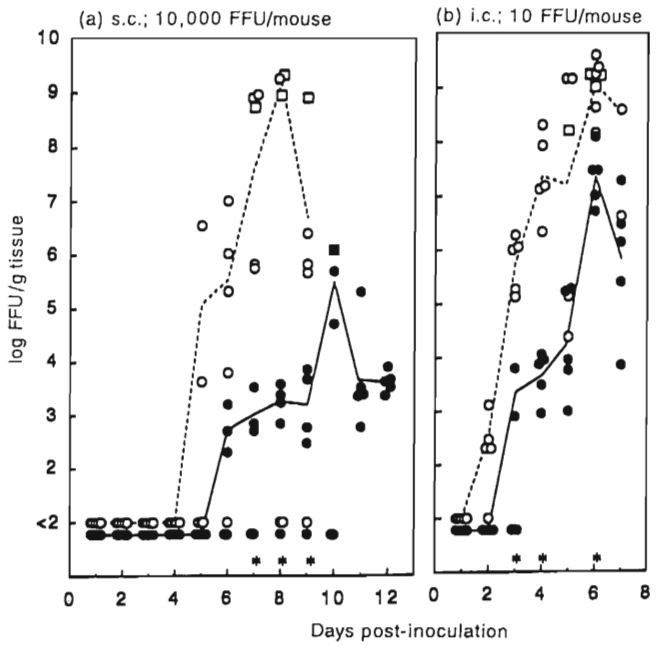

Fig. 8. Replication of TBE virus strains in mouse brain. Mice were inoculated subcutaneously (s.c.) with 10,000 FFU (a) or intracerebrally (i.c.) with 10 FFU (b) of TBE virus strains Oshima 5-10 (●; surviving, ■; dead) or Sofjin (○; surviving, □; dead), respectively. Virus titers in each mouse are shown. Geometric mean titers are calculated for each time period in Oshima 5-10 (solid line) and Sofjin (dotted line) strains, respectively. There were significant differences ($P < 0.05$) in geometric mean titers between the mice inoculated with Oshima and Sofjin strains on the days indicated by an asterisk.
(ref. 4)

2. Evaluation of European TBE vaccine

We tested the efficacy of European TBE vaccine against TBE virus, Hokkaido strain (5). Neutralizing antibody titers against TBE virus strains and JE virus strain in sera from healthy male volunteers vaccinated twice with FSME-IMMUN twice at a 1-month interval are shown in Table 7. Seroconversion rates to Oshima, Hochosterwitz, and Sofjin strains 4 weeks after the 2nd vaccination were 80, 100, and 90%, respectively; in addition, those rates obtained after the first vaccination were 60, 70, and 20%, respectively (data not shown). No significant differences were observed among the geometric mean titers (GMTs) (Oshima; 1:44, Hochosterwitz; 1:65, Sofjin; 1:43). Four of 10 persons were revaccinated 1 year later. After the 3rd vaccination, all persons showed two to four-fold increases in neutralizing antibody titer against at least some strains (Table 7). After the 2nd vaccination, two persons (Nos. 1 and 3) showed higher neutralizing antibody titers against JE virus, JaGAr-O1 strain, than those against Oshima strain (Table 7).

Groups of 10 mice were s.c. vaccinated with FSME-IMMUN and revaccinated 7 days later. GMTs against TBE virus strains in the sera obtained from the mice are shown in Table 8. With the exception of one individual showing an undetectable level of antibody against Sofjin strain, all of the mice seroconverted

Table 7. Neutralizing antibody titers in humans against TBE virus strains and JE virus strain after vaccination

	No.	Age (years)	NT titer against TBE virus: Oshima 5-10	TBE virus: Hochosterwitz	TBE virus: Sofjin	JE virus: JaGAr-O1
after 2nd vaccination	1[1]	49	<20[2]	20	<20	160
	2	42	<20	40	20	<20
	3[1]	29	20	20	20	40
	4	53	40	40	20	<20
	5	24	40	80	80	<20
	6	45	40	80	80	<20
	7	36	40	160	40	<20
	8	37	40	160	20	<20
	9	37	80	80	80	<20
	10	28	80	160	160	<20
GMT[3]			44±18	65±22	43±23	–
after 3rd vaccination	1	49	40	40	40	160
	2	42	80	160	80	<20
	4	53	40	80	40	<20
	8	37	160	160	80	<20

[1] Person who had NT antibody against JE virus (JaGAr-O1) before vaccination (No. 1; 1:160, No. 3; 1:20).
[2] Each titer was determined as the reciprocal of the highest serum dilution that reduced the virus focus counts by 50%.
[3] GMT; geometric mean titer of positive sera (mean±SD). (ref. 5)

Table 8. Neutralizing antibody titers in mice against TBE virus strains before and after infection

Virus strain	Immunogen	Pre-infection	Post-infection[1]
Oshima 5-10	PBS	<20 (0/10)[2]	320±16 (3/3)
	Vaccine	130±27[3] (10/10)	208±22 (8/8)
Hochosterwitz	PBS	<20 (0/10)	<20 (0/1)
	Vaccine	160±25 (10/10)	587±19 (8/8)*
Sofjin	PBS	<20 (0/10)	nt[4]
	Vaccine	127±30 (9/10)	80±17 (10/10)

[1]Sera were collected from surviving mice 21 days after infection. (ref. 5)
[2]( ); number of positive sera/number of mice tested.
[3]Geometric mean titer of positive sera (mean±SD).
[4]nt, not tested because none of the mice inoculated with Sofjin strain survived.
*; Significantly different from the pre-infection value ($P < 0.05$).

with GMTs of 1:130, 1:160, and 1:127 to Oshima, Hochosterwitz, and Sofjin strains, respectively. No significant differences were observed among GMTs similar to human vaccine trials.

The percentages of mice surviving after being vaccinated and challenged with 100 LD_{50} of each TBE virus strain are shown in Fig. 9. Vaccinated mice can be significantly protected against a challenge of TBE virus strains of Oshima, Hochosterwitz, and Sofjin. Notably, 100% protection was achieved after Sofjin infection. Neutralizing antibody titers against homologous strains were assayed 21 days after virus challenge in surviving mice. Although there were no significant differences in GMTs in sera of the vaccinated mice before or after challenge with Oshima and Sofjin strains, respectively, significant increases in GMT were observed in the sera of vaccinated mice after challenge with Hochosterwitz strain (Table 8). Moreover, GMT levels in the sera of control mice challenged with Oshima strain were 1:320, while these in the mouse challenged with Hochosterwitz strain were undetectable.

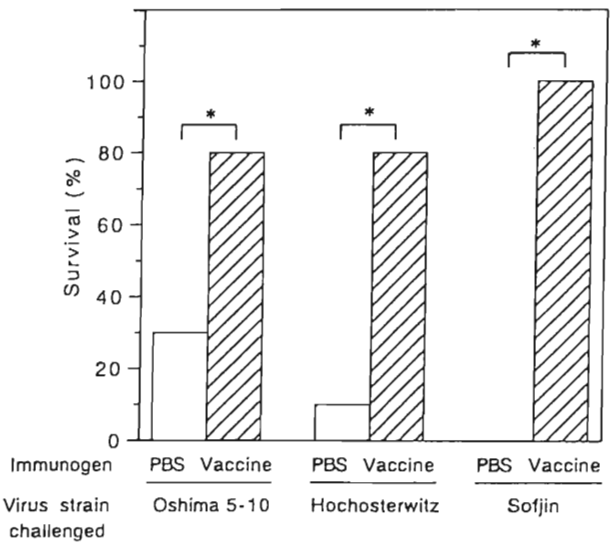

Fig. 9. Percentages of mice surviving after being vaccinated and challenged with 100 LD_{50} of TBE virus strains Oshima 5-10, Hochosterwitz, and Sofjin, respectively. Mice were subcutaneously immunized with phosphate buffered saline (PBS)(open bar) or European vaccine (hatched bar) twice at an interval of 1 week, and challenged by intraperitoneal inoculation of each TBE virus strain. Survival was recorded every day for 21 days after virus challenge. *; significant difference ($P < 0.05$). (ref. 5)

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REFERENCES

1. Ando, K., Kuratsuka, K., Arima, S., Hironaka, N., Honda, Y. and Ishii, K. (1952): Studies on the viruses isolated during epidemic of Japanese B encephalitis in 1948 in Tokyo area. Kitasato Arch. Exp. Med., 24, 49-61.
2. Calisher, C. H., Karabatsos, N., Dalrymple, J. M., Shope, R. E., Porterfield, J. S., Westerway, E. G. and Brandt, W. E. (1989): Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J. Gen. Virol., 70, 37-43.
3. Chambers, T. J., Hahn, C. S., Galler, R. and Rice, C. M. (1990): Flavivirus genome organization, expression, and replication. Ann. Rev. Microbiol., 44, 649-688.
4. Chiba, N., Iwasaki, T., Mizutani, T., Kariwa, H., Kurata, T. and Takashima, I. (1999): Pathogenicity of tick-borne encephalitis virus isolated in Hokkaido, Japan in mouse model. Vaccine, 17, 779-787.
5. Chiba, N., Osada, M., Komoro, K., Mizutani, T., Kariwa, H. and Takashima, I. (1999): Protection against tick-borne encephalitis virus isolated in Japan by active and passive immunization. Vaccine, 17, 1532-1539.
6. Dumpis, U., Crook, D. and Oksi, J. (1999): Tick-borne encephalitis. Clin. Infect. Dis., 28, 882-890.
7. Ecker, M., Allison, S. L., Meinxner, T. and Heinz, F. X. (1999): Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. J. Gen. Virol., 80, 179-185.
8. Hayasaka, D., Suzuki, Y., Kariwa, H., Ivanov, L., Volkov, V., Demenev, V., Mizutani, T., Gojobori, T. and Takashima, I. (1999): Phylogenetic and virulence analysis of tick-borne encephalitis viruses from Japan and far-eastern Russia. J. Gen. Virol., 80, 3127-3135.
9. Heinz, F. X., Kunz, C. and Fauma, H. (1980): Preparation of highly purified vaccine against tick-borne encephalitis

by continuous flow zonal ultracentrifugation. J. Med. Virol., 6, 213.
10. Korenberg, E. I. and Kovalevskii, Y. V. (1999): Main features of tick-borne encephalitis eco-epidemiology in Russia. Zentralbl. Bakteriol., 289, 525-539.
11. Kunz, C., Hofmann, H. and Stary, A. (1976): Field studies with a new tick-borne encephalitis (TBE) vaccine. Zentralbl. Bakteriol. Hyg. (A), 234, 141-144.
12. Kunz, C., Heinz, F. X. and Hofmann, H. (1980): Immunogenicity and reactogenicity of a highly purified vaccine against tick-borne encephalitis. J. Med. Virol., 6, 103-109.
13. Kunz, C., Hofmann, H., Heinz, F. X. and Dipple, H. (1980): Efficacy of vaccination against tick-borne encephalitis. Wien. Klin. Wochenschr., 92, 809-813.
14. Mandl, C. W., Holzmann, H., Meixner, T., Rauscher, S., Stadler, P. F., Allison, S. L. and Heinz, F. X. (1998): Spontaneous and engineered deletions in the 3'-noncoding region of tick-borne encephalitis virus: construction of highly attenuated mutants of a flavivirus. J. Virol., 72, 2132-2140.
15. Monath, T. P. and Heinz, F. X. (1996): Flaviviruses. p. 961-1034. *In* Fields, B. N., Knipe, D. M., Howley, P. M., et al. (eds.), Fields' Virology. 3rd ed. Vol. 1. Lippincott-Raven, Philadelphia.
16. Morita, K., Igarashi, A., Sato, T. and Takezawa, C. (1994): A case suspected as tick-borne encephalitis in Hokkaido, Japan. Infect. Agents Surveillance Rep., 15, 273-274 (in Japanese).
17. Pavlovsky, E. N. (1947): Ticks and tick-borne encephalitis. Parazitologia Dalnego Vostoka Leningrad, 5, 212-264.
18. 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.
19. Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. and Harrison, S. C. (1995): The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature, 375, 291-298.
20. Saeki, K. (1961): A neurotropic virus isolated from rodents trapped in the Hidaka area. Jpn. Med. J., No. 1611, 1247 (in Japanese).
21. Schneider, H. (1931): Uber epidemische akute Meningitis serosa. Wien. Klin. Wochenschr., 44, 350-352.
22. Takashima, I., Morita, K., Chiba, M., Hayasaka, D., Sato, T., Takezawa, C., Igarashi, A., Kariwa, H., Yoshimatsu, K., Arikawa, J. and Hashimoto, N. (1997): A case of tick-borne encephalitis in Japan and isolation of the virus. J. Clin. Microbiol., 35, 1943-1947.
23. Takeda, T., Ito, T., Chiba, M., Takahashi, K., Niioka, T. and Takashima, I. (1998): Isolation of tick-borne encephalitis virus from *Ixodes ovatus* (Acari: Ixodidae) in Japan. J. Med. Entomol., 35, 227-231.
24. Takeda, T., Ito, T., Osada, M., Takahashi, K. and Takashima, I. (1999): Isolation of tick-borne encephalitis virus from wild rodents and a seroepizootiologic survey in Hokkaido, Japan. Am. J. Trop. Med. Hyg., 60, 287-291.
25. Takezawa, C., Sato, T., Mizutani, Y., Abe, S. and Morita, K. (1995): Russian spring-summer encephalitis. A case report. Neurol. Med., 43, 251-252 (in Japanese).
26. Wengler, G., Bradley, D. W., Collett, M. S., Heinz, F. X., Schlesinger, R. W. and Strauss, J. H. (1995): Genus flavivirus. p. 426-427. *In* Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A., and Summers, M.D. (eds.), Virus Taxonomy; Classification and Nomenclature of Viruses. Springer-Verlag, Wien.
27. Zanotto, P. M., Gao, G. F., Gritsun, T., Marin, M. S., Jiang, W. R., Venugopal, K., Reid, H. W. and Gould, E. A. (1995): An arbovirus cline across the northern hemisphere. Virology, 210, 152-159.
28. Zilber, L. A. and Soloviev, V. D. (1946): Far Eastern tick-borne spring-summer (spring) encephalitis. Am. Rev. Sov. Med., Spec., 1-80.