

A BHK-21 cell culture-adapted tick-borne encephalitis virus mutant is attenuated for neuroinvasiveness

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Abstract

We derived the baby hamster kidney (BHK)-21 cell culture-adapted, tick-borne encephalitis (TBE) virus mutant. To reveal the pathogenicity of the TBE virus, we compared the pathogenicity of the mutant (Oshima CI-1) and parental (Oshima 5-10) virus in mouse model. The neurovirulence of mutant in mice was identical to that of parent. However, the level of neuroinvasiveness was higher for parent than for mutant. The degrees of viremia and virus titers in the spleen were lower in mice that were inoculated subcutaneously (s.c.) with mutant than in mice that received parent. Unlike parent, mutant was rarely detected in the brains of s.c. inoculated mice. Genetic analysis revealed that mutant had single amino acid substitutions in each of the E and NS5 proteins compared with parent. Furthermore, while mutant infection of BHK-21 cells was inhibited by glycosaminoglycans (GAGs), this was not the case for parent. In summary, the BHK-21-cell-adapted mutant virus showed reduced neuroinvasiveness in mice due to low-level induction of viremia. The attenuation process involved a single amino acid change in the E protein, which may have resulted in the rapid clearance of the virus due to its high affinity for negatively charged molecules *in vivo*.

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Keywords: Tick-borne encephalitis virus; Attenuation; Cell-adapted mutant virus

1. Introduction

Tick-borne encephalitis (TBE) virus, which is a member of the genus *Flavivirus* in the family *Flaviviridae*, causes fatal encephalitis in humans [10]. TBE virus is a single-stranded, positive-polarity, enveloped RNA virus. The RNA genome of TBE virus is about 11 kb in length. A single large open reading frame encodes three structural proteins [core (C), membrane (prM), and envelope (E) proteins] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The 5'- and 3'-ends of the viral genome are non-coding regions (NCRs) [6].

Following subcutaneous inoculation of encephalitogenic flaviviruses, which include the TBE virus, virus replication is first detected within the draining lymph nodes, and plasma viremia ensues [27]. Many extraneural tissues are infected during viremia and the release of viruses from these tissues ensures that viremia persists for several days [1,27,29]. In

terms of extraneural tissues, the virus is detected mainly in the spleen [18], and virus entry into the brain occurs during the viremic phase [27]. The ability to replicate in extraneural tissues after peripheral inoculation, to induce viremia, and to invade the central nervous system (CNS) is referred to as “neuroinvasiveness” [26,29]. The means by which encephalitogenic flaviviruses gain entry into the CNS has been debated for many years and remains unsolved. Although the blood–brain barrier was believed to be the port of entry route for the virus, McMinn et al. [27] suggested that encephalitogenic flaviviruses enter the murine CNS via the olfactory pathway.

The low neuroinvasiveness of encephalitogenic flaviviruses may be due to low levels of virus replication or spread in peripheral tissues and poor capacity to enter the brain. Several studies have established that single amino acid changes in critical determinants of the envelope (E) glycoprotein, which is found on the viral surface and associates with the viral envelope, are sufficient to cause loss of neuroinvasiveness [5,14,15,16,20,21,23,26–28]. The attenuated virulence of these variants is probably due to a change in the ability of the E protein to mediate fusion and

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receptor binding to critical cell types within the peripheral tissues of the host. An understanding of the molecular genetic basis for virulence is essential for the development of live attenuated vaccines.

Serial passage of viruses in cell culture sometimes produces cell-adapted mutant viruses. Some of these mutants show changes in the net positive charge of their E proteins and they have high affinity for glycosaminoglycans (GAGs), which are highly-sulfated polysaccharides that are present almost ubiquitously on cell surfaces [22]. It has become clear that viruses from many different families interact with GAGs. Thus, viral affinity for GAGs may be an important determinant of tissue tropism and pathogenicity [3,19–23,34]. Recently, Mandl et al. [23] described BHK-21-cell-adapted mutants of TBE virus that had increased affinity for GAGs. There are many reports that virus adaptation to cell lines results in reduced virulence *in vivo*. However, the mechanism by which cell-adapted flaviviruses undergo attenuation *in vivo* is unclear.

In this study, we derived the BHK-21-cell-adapted TBE virus mutant. To reveal the attenuation mechanism of the TBE virus, we compared the neurovirulence and neuroinvasiveness of the mutant and parental virus in mice. We also compared the levels of viremia, virus growth in the spleen, and antibody responses among mice that were inoculated with the two viruses. In addition, the biological properties and the deduced amino acid sequences of the two viruses were compared. The correlation between viral virulence and observed characteristics is discussed.

2. Materials and methods

2.1. Cell lines and viruses

The baby hamster kidney (BHK)-21 cell line (ICN Biomedicals, Aurora, OH, USA) was grown at 37 °C in Eagle's Minimum Essential Medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) that contained 8% fetal calf serum (FCS; ICN Biomedicals). The TBE viruses used included the Oshima 5-10 strain (Far-Eastern subtype TBE virus isolated from dog blood in Hokkaido, Japan) [8,35], and the Oshima CI-1 mutant (passed twice on BHK-21 cells from Oshima 5-10 and cloned three times and selected on the basis of forming larger plaques on BHK-21 cells). The brains of virus-inoculated 1-day-old suckling mice were homogenized and diluted in 1% phosphate buffered saline (PBS; pH 7.6) that contained 10% FCS, and stored at –80 °C for use as virus stocks. The infectious virus titer was assayed by the focus count method, as described previously [35]. Briefly, monolayers of BHK-21 cells were grown in 96- or 24-well plates and inoculated with serial dilutions of the virus. After incubation at 37 °C for 38 h, viral foci in the cell monolayers were visualized by immunohistochemical staining using the peroxidase–antiperoxidase (PAP) procedure [17].

2.2. Plaque formation assay

Monolayers of BHK-21 cells were grown in six-well plates and infected with serial dilutions of the virus in culture medium. After incubation at 37 °C for 90 min, the cells were washed twice with PBS, and overlaid with 2 ml MEM that contained 10% FCS and 7% agarose (SEAKEM ME; FMC BioProducts, Rockland, ME, USA). At 96 h postinfection (p.i.), the cells were stained with 5% neutral red in 2 ml MEM that contained 10% FCS and 7% agarose. Plaque morphology was observed after incubation for 24 h.

2.3. Growth curve

Monolayers of BHK-21 cells grown in 24-well plates were infected with virus in culture medium at a multiplicity of infection (MOI) of 1 focus-forming unit (FFU). After incubation at 37 °C for 90 min, cells were washed twice with PBS and 1 ml of MEM containing 4% FCS was added. At 6, 9, 12, 18, 24, 30 and 36 h p.i., samples were harvested from the cell culture medium, stored at –80 °C and titrated by focus count assay for quantification of the infectious virus titer.

2.4. Virulence assays

Virulence assays were performed in 8-week-old male ICR mice (SLC, Shizuoka, Japan). Groups of 10 mice were inoculated either subcutaneously (s.c.) with 10,000 FFU or intracerebrally (i.c.) with 10 FFU of virus. Water and pellet diets were supplied *ad libitum*. The survival rates of the infected mice were observed and recorded daily for a period of 28 days p.i. The animals were infected and handled under P3 containment conditions.

2.5. Viral replication in mouse organs

Male 8-week-old ICR mice were inoculated s.c. with 10,000 FFU of each viruses. At the indicated times after inoculation, five mice from each group were killed by exsanguination via cardiac puncture under anesthesia, and the blood, brains, and spleens were collected. All samples were individually weighed and stored at –80 °C. The samples were subsequently homogenized in 10% (brains and spleens) or 50% (blood) suspensions in PBS that contained 10% FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The virus titers were measured using the focus count assay. The thresholds for viral detection were approximately 200 and 50 FFU/g in 10% tissue suspensions and 50% blood suspensions, respectively.

Serum samples were also taken from the mice, stored at –80 °C and subsequently examined for TBE-specific antibodies using the neutralization test (NT).

2.6. Neutralization tests with mouse sera

The NT was performed using the procedure that was described previously [17]. Briefly, serially diluted sera

(50 μ l) were mixed with equal volumes of stock virus (100 FFU/50 μ l) and the mixtures were incubated at 37 °C for 90 min. Monolayers of BHK-21 cells were grown in 96-well plates and inoculated with 50 μ l per well of the mixtures and incubated at 37 °C for 90 min. The viral inocula were then removed, the plates were rinsed three times with PBS, and MEM containing 1.5% carboxymethyl cellulose and 4% FCS (CMC–MEM; 110 μ l per well) was layered onto the cells. The monolayers were then incubated in a CO₂ incubator at 37 °C for 38 h. The foci of virus-infected cells were visualized using the PAP procedure, as described above. The neutralizing antibody titer was determined as the reciprocal of the highest serum dilution that reduced the viral foci counts by 50% or more.

2.7. Genome sequence analysis

The nucleic acid sequences of the viral genomes were determined by direct sequencing of the RT-PCR products. Viral RNA was extracted from the brains of virus-inoculated suckling mice using an Isogen Kit (Nippon Gene, Tokyo, Japan). For cDNA synthesis, PCR was performed using a THERMOSCRIPT™ RT-PCR System Kit (Invitrogen, Carlsbad, CA, USA). The cycle sequencing reactions were performed using a DNA Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and the sequences were determined with an ABI PRISM 310 fluorescence autosequencer (Applied Biosystems). Alignments of the nucleic acid sequences and deduced amino acid sequences were performed with GENETYX-MAC version 10 (Software Development, Tokyo, Japan).

2.8. Hemagglutination assay

The hemagglutination assay was performed as described previously [9]. Briefly, sucrose–acetone-extracted antigen was prepared from the supernatants of virus-infected BHK-21 cells, and used as the source of HA. Goose erythrocytes were collected in acid–citrate dextrose solution, washed in PBS, resuspended as a 10% solution in dextrose–gelatin–veronal, and stored at 4 °C. HA was serially diluted in borate-buffered saline (pH 9.0) in 96-well microtiter plates. Hemagglutination was assayed over pH range 6.0–7.0, and the optimum pH for hemagglutination was determined as the pH that caused complete agglutination of goose erythrocytes by the highest dilution of sucrose–acetone-extracted viral antigen.

2.9. GAG inhibition of virus infectivity

The GAG inhibition assays were carried out with heparin and chondroitin sulfates A–C (Sigma–Aldrich, St. Louis, MO, USA).

Serially diluted GAGs (50 μ l) were mixed with equal volumes of stock virus (100 FFU/50 μ l) and the mixtures were incubated at 4 °C for 30 min. Monolayers of BHK-21 cells

were grown in 96-well plates, inoculated with the mixture (50 μ l per well), and incubated at 37 °C for 90 min. The mixture was removed and the plates were rinsed twice with PBS, CMC–MEM (110 μ l per well) was layered onto the cells. After incubation at 37 °C for 38 h, viral foci in the cell monolayers were visualized by immunohistochemical staining with PAP. The GAG-mediated inhibition rate was determined as follows: $[1 - (\text{the number of viral foci at the designated GAG concentration} / \text{the number of viral foci in the absence of GAG})] \times 100\%$.

2.10. Statistical analysis

The survival rates of mice that were inoculated s.c. or i.c. with Oshima CI-1 or Oshima 5-10 were analyzed using the Fisher's exact probability test. The survival times of mice that were inoculated s.c. or i.c. with two viruses, and differences in viral replication in mouse organs between the two viruses were analyzed using the Mann–Whitney *U*-test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Plaque formation assay and growth curve

Oshima CI-1 mutant was generated by selecting a large plaque after five rounds of subculturing Oshima 5-10 strain in BHK-21 cells. We examined the plaque morphologies of both viruses in BHK-21 cells (Fig. 1). BHK-21 cells in 6-well plates were infected with 50 FFU per well of each TBE virus, and the plaques were observed at 120 h p.i. The plaques of Oshima CI-1 on BHK-21 cells were larger than those of Oshima 5-10 (Fig. 1A).

To identify differences in viral replication between the two viruses, the infectious virus titer of culture fluid harvested at various time points was assayed by the focus count method (Fig. 1B). Virus titers in the culture fluid of Oshima CI-1 increased at 9 h p. i. In contrast, those of Oshima 5-10 remained low until 9 h p.i. and then the titer increased at 12 h p.i., but to the level of 1/100 of Oshima CI-1. This result reveals that mutant Oshima CI-1 replicates more rapid than strain Oshima 5-10.

3.2. Virulence tests in the animal model

The degree of neuroinvasiveness of the viruses was determined by subcutaneous viral inoculation into mice to mimic the natural mode of TBE infection, i.e. virus spread from a peripheral infection site in the skin. Mice were also inoculated i.c. to evaluate the degree of neurovirulence.

Subcutaneous inoculation of mice with 10,000 FFU of each virus revealed significant differences in neuroinvasiveness between the two viruses (Fig. 2A). Eighty percent of the mice that were inoculated with Oshima CI-1 survived, but only 30% of those inoculated with Oshima 5-10 survived.

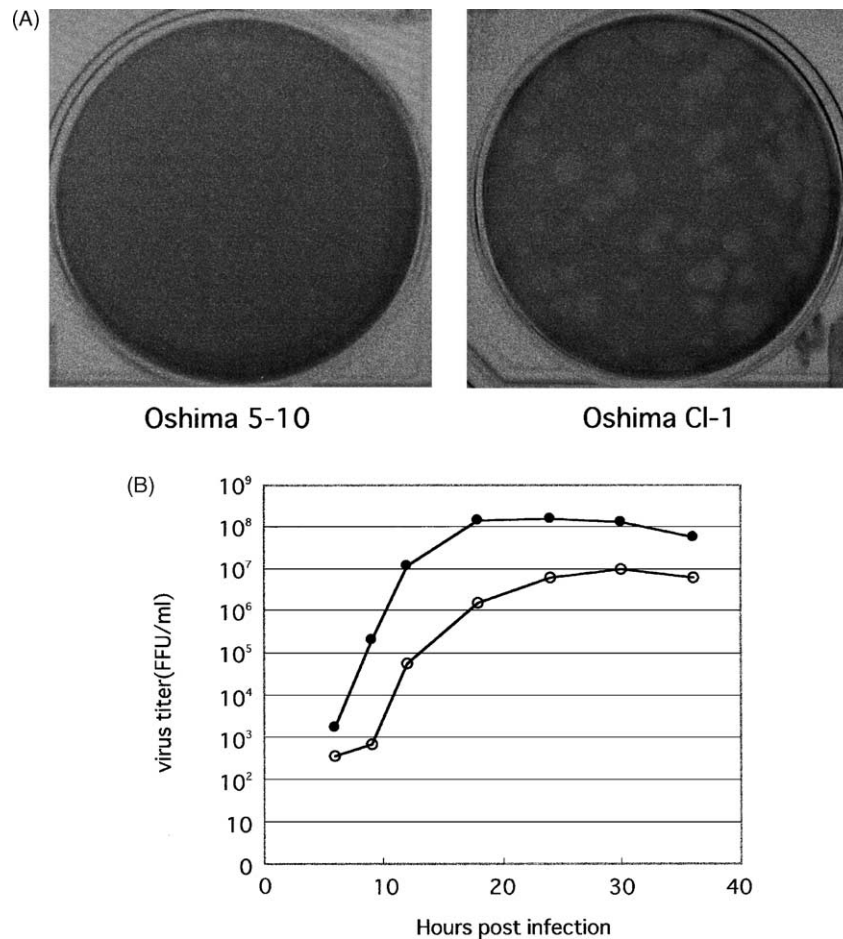


Fig. 1. Growth of TBE virus in BHK-21 cell culture. (A) Plaque morphology on BHK-21 cells at 120h p.i. BHK-21 cells were infected with Oshima 5-10 and Oshima CI-1. (B) Growth curve. BHK-21 cells were infected at a MOI of 1 FFU of either Oshima CI-1 (●) or Oshima 5-10 (○). The titer of virus released during each time interval was determined by focus counting assay on BHK-21 cell.

Moreover, the survival times of mice that were inoculated with Oshima CI-1 were significantly longer than those of mice that received Oshima 5-10 ($P < 0.05$).

The neurovirulence levels of the two viruses were determined by intracerebral inoculations of 10 FFU (Fig. 2B). The survival times of mice that were inoculated i.c. with Oshima CI-1 were longer than those of mice that received Oshima 5-10 ($P < 0.05$). However, no significant difference was observed between the rates of the survived mice that inoculated with Oshima CI-1 or Oshima 5-10 (30 and 0%, respectively).

In summary, the degree of neuroinvasiveness of Oshima CI-1 was significantly lower than that of Oshima 5-10, although the levels of neurovirulence were almost similar between the two viruses.

3.3. Virus replication and neutralizing serum antibody responses of TBE virus-inoculated mice

The low-level neuroinvasiveness of Oshima CI-1 may be due to reduced virus replication or to spread in peripheral

tissues and reduced capacity to enter the brain. In order to examine these issues, the levels of viral replication in the blood, brain, and spleen were compared in mice that were inoculated s.c. with the Oshima 5-10 and Oshima CI-1.

After inoculation with Oshima 5-10, viremia was first detected 1 day p.i., peaked 3 days p.i. (1×10^3 FFU/ml; the average of five samples), decreased gradually, and was undetectable by 5 days p.i. (Fig. 3A). Virus was detected in the spleen samples 2 days p.i., and the titers reached 5×10^3 FFU/g at 5 days p.i. Virus was hardly detectable in the spleens from 11 days p.i. (Fig. 3B). Virus was first detected in the brain tissues at 7 days p.i., and the titers reached the average 2×10^4 FFU/g at 9 days p.i. (Fig. 3C). Oshima 5-10 infected mice developed clinically apparent encephalitis at 7 days p.i. The neutralizing antibody titer in the serum was first detected 4 days p.i., and the peak titer was noted at 9 days p.i. (Fig. 4).

On the other hand, after inoculation with Oshima CI-1, viremia was first detected at 2 days p.i. (60 FFU/ml; the average of five samples), decreased low level, and was undetectable by 3 days p.i. (Fig. 3A). The virus was first detected

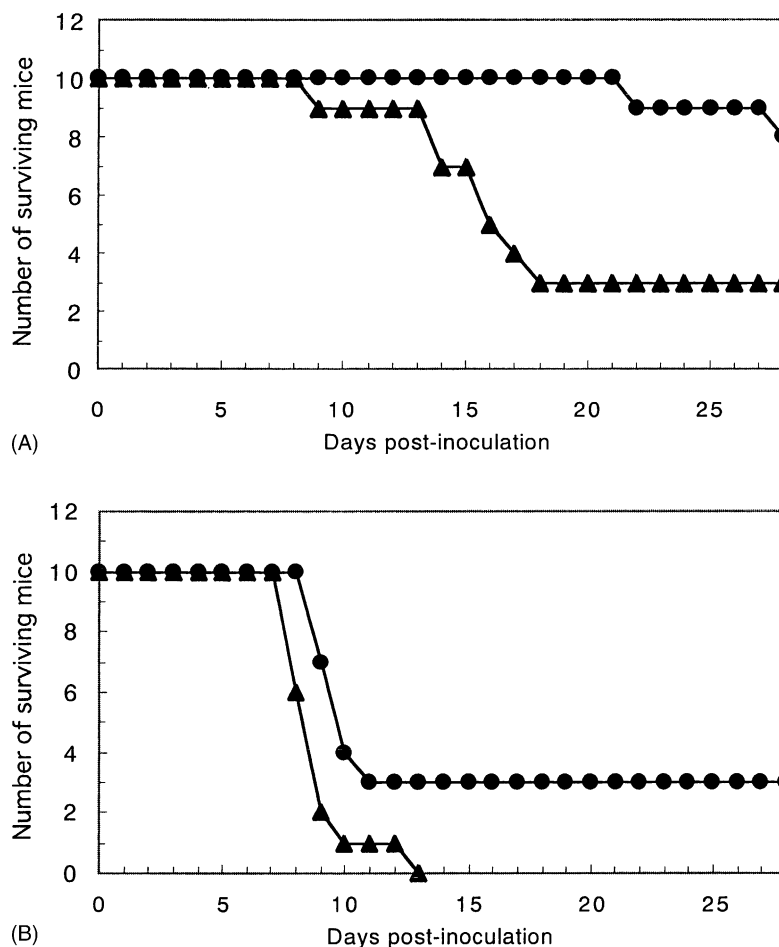


Fig. 2. Survival of adult mice that were inoculated with TBE virus. The mice were inoculated subcutaneously with 10,000 FFU (A) or intracerebrally with 10 FFU (B) of TBE virus strains Oshima CI-1 (●) or Oshima 5-10 (▲), respectively.

in the spleen samples at 3 days p.i., and the titers peaked at 5 days p.i. (5×10^2 FFU/g). The virus was not detectable in the spleen from 9 days p.i. (Fig. 3B). Virus was not detected in the brain tissues by 11 days p.i. (Fig. 3C), and none of the mice developed clinically apparent encephalitis during the period of observation. The neutralizing antibody in the serum was first detected 7 days p.i., and the peak titer was noted at 9 days p.i. (Fig. 4).

Collectively, there were no differences in antibody responses between mice that were inoculated with Oshima 5-10 or Oshima CI-1 (Fig. 4). However, the peak viremia titers of the mice that were inoculated s.c. with Oshima CI-1 were 100-fold lower than those of mice that received Oshima 5-10 (Fig. 3A). The virus titers in the spleens of mice that were inoculated with Oshima CI-1 were also 10-fold lower than those of Oshima 5-10-inoculated mice (Fig. 3B). Furthermore, viral entry into the brain was observed at 7 days p.i. in the mice that were inoculated with Oshima 5-10, whereas negligible amounts of virus were detected in the brains of mice that were inoculated with Oshima CI-1 (Fig. 3C).

3.4. Genome sequence analysis

Nucleotide sequence analysis revealed that the genomes of both Oshima 5-10 and Oshima CI-1 contained 11,100 nucleotides. Oshima CI-1 differed by a total of three nucleotides (0.1%) and two amino acids (0.1%) from Oshima 5-10 (Table 1). One of the two amino acid substitutions was detected in the NS5 protein, and represented a conservative change that did not affect the residue characteristics, such as charge and polarity [36]. However, the other substitution, which was detected in the E protein, increased the overall positive charge of the E protein of Oshima CI-1. This substitution was located on domain II of the E protein [32] and previous studies showed that mutation in this domain had significant effects on fusion and/or hemagglutination (HA) activities [5,14,16,27,28]. We compared the HA activities of the two viruses to see whether this substitution influenced the function of domain II. We found that both Oshima CI-1 and Oshima 5-10 were able to hemagglutinate at their optimal pH levels (pH 6.6 and pH 6.4, respectively; data not shown).

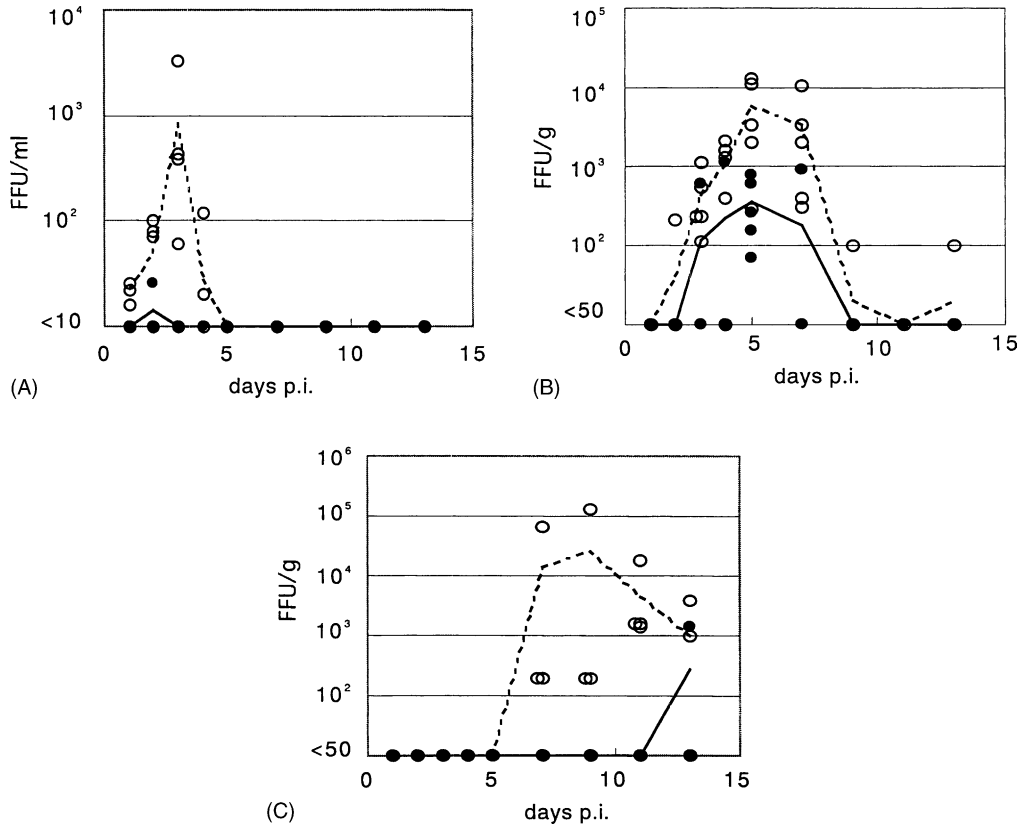


Fig. 3. Kinetics of TBE virus titers in the blood and tissues of mice (blood, (A); spleen, (B); brain, (C)). Mice were inoculated subcutaneously with 10,000 FFU of TBE virus strains Oshima CI-1 (●) or Oshima 5-10 (○). The individual virus titers are shown. Geometric mean titers were calculated for each time period for Oshima CI-1 (solid line) and Oshima 5-10 (dashed line).

3.5. GAG inhibition of virus infectivity

Viral adaptation to certain cell lines results in the selection of mutants that bind GAGs with high affinity, and

some of these viruses have reduced virulence in animals [3,19–23,34]. To compare the affinities for GAGs of our two viruses, we examined the inhibitory effects of different GAGs on viral infectivity.

Infectivity of Oshima 5-10 for BHK-21 cells was rarely inhibited by GAGs (Fig. 5A). In contrast, the infectivity of Oshima CI-1 was inhibited by all of the GAGs tested, and the inhibition was dose-dependent (Fig. 5B). The rates of inhibition of infectivity by heparin and chondroitin sulfate B were almost 100% at low concentrations of GAGs (31.25 μg/ml), and chondroitin sulfate C inhibited virus infection by a maximum of 70%. However, the highest concentration of chondroitin sulfate A inhibited viral infectivity by only 20% (Fig. 5B).

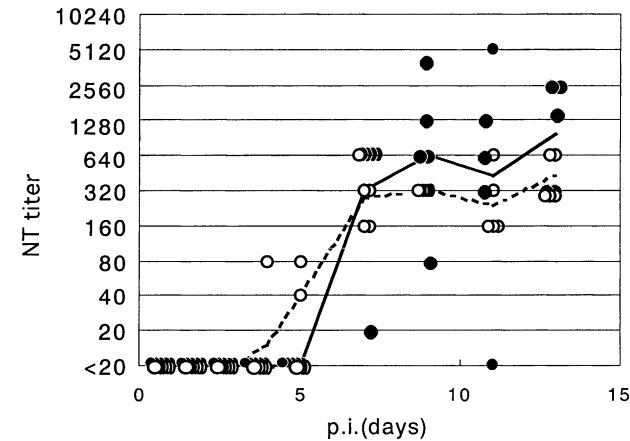


Fig. 4. Development of neutralizing anti-TBE antibody titers in mice. Mice were inoculated subcutaneously with 10,000 FFU of TBE virus strain Oshima CI-1 (●) or Oshima 5-10 (○). The individual antibody titers are shown. Geometric means of the neutralizing antibody titers were calculated for each time period for the Oshima CI-1 (solid line) and Oshima 5-10 (dashed line) strains.

4. Discussion

Extensive information on viral virulence is essential for understanding the disease process and for the development of live attenuated vaccines. In the case of flaviviruses, some of that cause very severe encephalitis, the mechanism by which the virus invades the CNS has not been fully elucidated because studies correlating in vivo virus dynamics and in vitro virus characteristics have been lacking. In this

Table 1
Nucleotide and amino acid differences between Oshima 5-10 and Oshima CI-1

	Nucleotide		Amino acid	
	Substitution (position no. ^a)	Percentage identity	Substitution (position no. ^a)	Percentage identity
E	A → G (1579)	99.9	Asp → Gly (483)	99.8
NS5	A → G (10228)	99.9	Lys → Arg (3366)	99.9
3'-NCR	C → T (10796)	99.9	–	–
Total		99.9		99.9

^a Numbers are according to the TBE virus Oshima 5-10 strain genomic sequence (GenBank Accession no. AB062063 [11]).

study, we explored flavivirus pathogenicity, paying particular attention to the neurovirulence and neuroinvasiveness in the mouse model of the BHK-21 cell-adapted mutant Oshima CI-1 and the parental Oshima 5-10 strain, which is a

Far-Eastern subtype TBE virus. To further explain the results from the mouse model in genetic and biochemical terms, we compared the characteristics of the mutant and the parent by sequence analysis and infectivity inhibition assays with GAGs.

The main finding of this study is that a single amino acid substitution in the viral E protein results in reduced neuroinvasiveness in the mouse model. The reduced neuroinvasiveness of the mutant was evident in the difference in survival rates of mice that were s.c. inoculated with 10,000 FFU of the mutant or parental strain (Fig. 2A). On the other hand, neurovirulence, as revealed by the survival rate after intracerebral inoculation of 10 FFU, was similar for both viruses (Fig. 2B). Sequence analysis of the mutant virus showed two amino acid substitutions in the coding region and one nucleotide substitution in the 3'-NCR gene (Table 1). Since the substitution in the E protein is non-conservative, while that on the NS5 protein is conservative [36], we believe that the mutation in the E protein is more important than that in the NS5 protein with respect to *in vivo* viral attenuation. Furthermore, previous studies showed that 3'-NCR gene defined the secondary structure, and deletion or alteration of this structure impaired virus replication [6,12,24]. However, in this study, the position of the substitution in the 3'-NCR did not affect the secondary structure of the 3'-NCR (data not shown), and we conclude that the mutation in the 3'-NCR had less influence on viral attenuation.

The reduction in the neuroinvasiveness of the mutant appears to be related to the low level of viremia and/or virus growth in peripheral organs, such as the spleen, when compared with the parental strain. The results show that the viremic levels and virus titers of the mutant in mouse spleens were 1/100 and 1/10, respectively, of those of the parental strain. Although the entry mode of flavivirus into the brain remains uncertain, several studies support the concept of hematogenous spread into the CNS and suggest a relationship between the level of viremia and the degree of brain infection [1,37]. Lee and Lobigs [21] recently showed similar data in mouse infection model with Japanese encephalitis virus and Murray Valley encephalitis virus. Mandl et al. [23] recently reported TBE virus mutants that were adapted in BHK-21 cells and attenuated for neuroinvasiveness in adult mice, but they did not compare the *in vivo* dynamics of the mutant and parental strains.

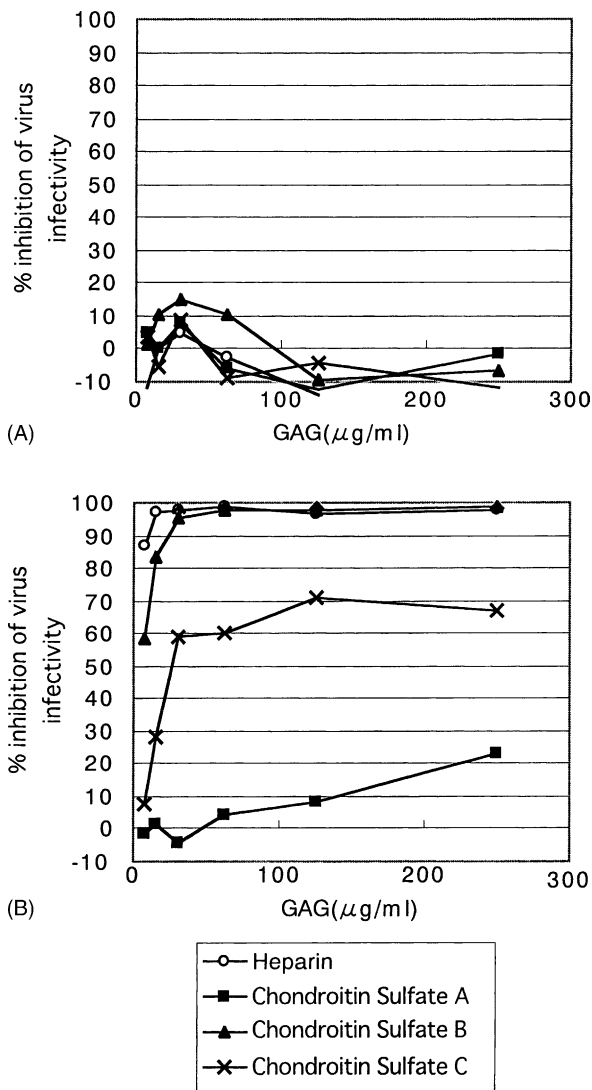


Fig. 5. Inhibition by GAGs of infections with the Oshima 5-10 (A) and Oshima CI-1 (B) viruses. Rates of inhibition were calculated according to the following formula: $1 - (b/a)$, where a was the number of foci on cells that were incubated with untreated virus, and b was the number of foci on cells that were incubated with GAG-treated virus.

The development of immune responses is important in determining neuroinvasiveness, as well as viral dissemination in host tissues. Several studies in animal models have suggested that humoral immune responses are more important than cell-mediated immunity in terms of protection and recovery from flavivirus encephalitis [4,27]. The difference in neuroinvasiveness observed between Oshima 5-10 and Oshima CI-1 in this study cannot be attributed to difference in antibody responses. The viremia levels in Oshima 5-10-inoculated mice appeared to be sufficient for viral invasion of the brain before the development of neutralizing antibodies, i.e. 4–5 days postinoculation. On the other hand, although the viremia levels in Oshima CI-1-inoculated mice were not high enough for neuroinvasion, virus growth in the spleens of these mice may have elicited adequate antibody responses.

The increased positive charge of the envelope protein of Oshima CI-1, which is due to a single amino acid substitution, may have resulted in low-level viremia and affected neuroinvasive virulence. It has been shown that increased positive charge on envelope protein results in high affinity of the virus for negatively charged substances, such as GAGs. We showed that Oshima CI-1 had much higher affinity than Oshima 5-10 for GAGs, and the rate of inhibition of infection by GAGs was dependent on the degree of GAG sulfation (Fig. 5). This result clearly shows that the E protein of Oshima CI-1 carries an increased positive charge compared to that of Oshima 5-10. Previous studies showed that GAG-adapted viruses had reduced virulence in animals [3,19–23,34]. The general conclusion from these studies is that virus dissemination is impaired and clearance from the circulation is accelerated in the natural host. For example, Lee and Lobigs [21] showed that the heparan sulfate-adapted Japanese encephalitis virus and Murray Valley encephalitis virus were deposited in the various organs, cleared rapidly from the blood of mice and had low-level neuroinvasiveness. In view of the previous data and our present data, we speculate that the more positively charged E protein binds ubiquitous, negatively charged glycoproteins *in vivo* and leads to a more rapid clearance of the virus from the blood of mice, which in turn reduces the neuroinvasive virulence of neuropathogenic viruses, such as flaviviruses and alphaviruses.

The amino acid substitution in the E protein of Oshima CI-1 was located in domain II, which constitutes the fusion peptide and has the ability to bind target membranes [15,32,33]. Another possible mechanism for the low level of viremia is the alteration of the low-pH induction of E protein function, as assessed by HA and fusion activities. In flaviviruses, fusion and HA activities depend on low-pH-triggered, dimer–trimer conformational changes in the E protein [2,13,15]. Previous studies have showed that mutations in domain II had significant effect on fusion and/or HA activities [5,14,16,27,28]. However, our results show that both Oshima CI-1 and Oshima 5-10 hemagglutinated goose erythrocytes at the optimum pH (data not shown). Therefore, the amino acid substitution in Oshima

CI-1 seemed to have no effect on HA activity and probably did not affect fusion activity.

Oshima CI-1 grew well in cell culture (Fig. 1) and its infectivity was inhibited following the addition of GAGs (Fig. 5B), which suggests that attachment to GAGs provides a decisive selective advantage in cell culture. It has been demonstrated that viral adaptation to certain cell lines often results in mutants that bind GAGs with high affinity [3,19–23,34]. Controversy persists as to whether GAGs serve as the sole receptors for flaviviruses or other molecules participate in this process [7,23,25]. Experiments to explore these possibilities have been done mainly in cell cultures. If we consider the perpetuation of flaviviruses in nature, mutants with high affinity for GAGs, such as Oshima CI-1, are unlikely to be maintained successfully among natural hosts, such as ticks and vertebrates. Although Oshima CI-1 was favorable for growth in BHK-21 cell cultures, its viremic level in mice was very low. Therefore, GAG-adapted mutants, such as Oshima CI-1, may be maintained only *in vitro* and may not support the transmission cycle in nature.

Oshima CI-1 showed reduced neuroinvasiveness but elicited good antibody responses in mice. These characteristics are good markers for a live attenuated vaccine. However, Oshima CI-1 is not suitable as a vaccine candidate strain due to its other characteristics. Oshima CI-1 had practically the same neurovirulence potential as Oshima 5-10, and could invade the brain at very low frequency (Fig. 3C). The neuroinvasive virulence of Oshima CI-1 may revert to that of the parent, since the attenuation is due to only one amino acid substitution in the E protein, which has an important role in viral infection. In contrast, the widely used live attenuated vaccine strains of yellow fever virus [31] and Japanese encephalitis virus [30] contain multiple point mutations, not only in E protein but elsewhere in the genome. Our results suggest that the *in vivo* characteristics of Oshima CI-1 are good markers for attenuation of the TBE virus, and encourage further study to understand the virulence of the virus and to develop a live attenuated vaccine.

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