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Comparison of the neuropathogenicity of two SSPE sibling viruses of the Osaka-2 strain isolated with Vero and B95a cells

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Two sibling viruses, Fr/V and Fr/B, of the subacute sclerosing panencephalitis (SSPE) virus Osaka-2 strain were isolated from a small biopsy specimen of the brain of an SSPE patient by cocultivation with two different cell lines, Vero and B95a cells, respectively. These two sibling viruses differ from each other in their molecular mechanisms of defective M protein expression. In this study, we found that the Fr/B virus could scarcely form syncytium foci on Vero cells, although the Fr/V virus could do so on both Vero and B95a cells, showing a similar relation of cell tropism between recent field isolates and laboratory strains of the measles virus. Severe neurovirulence of both Fr/V and Fr/B viruses was observed in hamsters inoculated intracerebrally with less than 100 PFU, in contrast to the negative neurological and pathological findings in hamsters inoculated even with more than 10^5 PFU of their possible progenitor measles virus. Comparative sequence analysis of inoculated viruses and reisolated viruses from diseased hamster brains showed few variations at a region containing the P-M gene junction, indicating that the inoculated viruses propagated in the brains and induced neurovirulence. All these results suggest that SSPE virus isolated with a lymphoid cell line is similar in neuropathogenicity to that isolated with a nonlymphoid cell lines, irrespective of differences in the molecular mechanism of M protein defectiveness. *Journal of NeuroVirology* (2002) **8,** 6–13.

Keywords: SSPE virus; neurovirulence; reisolated virus

Introduction

Subacute sclerosing panencephalitis (SSPE) is a degenerative and fatal disease of the central nervous system (CNS) acquired by children around 7 years of age after contracting measles. Almost all of these children have suffered from measles a year or two after birth. SSPE is rare, but no curable treatment has yet been established. Measles virus variants, referred to as the SSPE viruses, can be isolated from the brain of SSPE patients.

We have isolated three strains (Osaka-1, -2, and -3) of infectious SSPE virus from specimens of brain biopsy or autopsy of patients with SSPE in Osaka (Ogura *et al*, 1997) and biochemically and genetically characterized the defectiveness of the Osaka-1 (Ayata *et al*, 1998a) and Osaka-2 strains (Seto *et al*, 1999) to allow for a better understanding of their pathogenicity, as there are numerous variants, most of which are nonreplicable or nonpathogenic, in SSPE brains. To specify the exact mutation characteristics of the SSPE viruses, we compared matrix (M) gene sequences of

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the three strains to those of their possible progenitor measles viruses isolated from measles patients from the same area and who contracted the disease during the same time period as the previous SSPE patients did (Ayata *et al*, 1998b).

The neuropathogenesis of SSPE viruses is of great importance. Measles viruses generally do not induce encephalitis by intracerebral inoculation in experimental animals except for brain-adapted strains in newborn animals (Baringer and Griffith, 1970; Albrecht and Schumacher, 1971; Byington and Johnson, 1972; Griffin *et al*, 1974). In contrast, SSPE viruses show extremely strong neurovirulence (Johnson and Byington, 1971; Katz *et al*, 1976; Albrecht *et al*, 1977; Johnson and Byington, 1977; Thormar *et al*, 1977). This neurovirulence has, however, been observed to vary widely among studies, even with the same strains (Ohuchi *et al*, 1981; Sakaguchi *et al*, 1985). These differences might be due to mutations during *in vitro* passages of the strains or to selection during multiplication of the strains in infected brains, which seems to be an unavoidable difficulty in the analysis of the neuropathogenesis of RNA viruses. One way of overcoming this obstacle is to compare virus growing in brains to inoculated virus at the genetic level. Cathomen *et al* (1998) recently showed that measles virus acquired enhanced cell fusion and slow propagation in the brain by knocking out M gene and shortening cytoplasmic tails of glycoproteins, suggesting that enhanced cell fusion might possibly play an important role for expression of subacute neuropathogenicity.

We have recently reported two sibling viruses, Fr/V and Fr/B, of the Osaka-2 strain isolated from a small piece of biopsy specimen with different cell lines, Vero cells and B95a cells, respectively (Ogura *et al*, 1997). These two viruses show different transcriptional patterns of the M genes in defective M protein expression (Seto *et al*, 1999). In this study, therefore, we investigated the influences of these differences on neuropathogenicity in hamsters; in addition, we confirmed the near identity of growing virus in diseased brains with inoculated virus based on genetic analysis of both viruses.

Results

Cell tropism of the sibling viruses to Vero or B95a cells

The Fr/V virus- and Fr/B virus-infected cells could not produce infectious cell-free virus due to defective M protein expression, which is different in molecular mechanism between the two sibling viruses (Seto *et al*, 1999), as summarized in Table 1. When hamsters were inoculated intracerebrally with virus-infected cells, the results were not quantitative because of the difficulty in reproducibly preparing the same number of infected cells. To inoculate the hamsters with more exact titers, we prepared cell-free pseudovirions by treating the virus-infected cells with cytochalasin D according to the method described by Ayata (1987). The infectivity in the supernatants of the cytochalasin D-treated cultures resisted to freezing at -20, -70, and -120°C and was reduced to about 10^{-3} by passing through a membrane filter with a pore size of 0.22 μm similarly to that of measles virus. Just like the original nonproductive SSPE virus, these particles showed only cell-associated infectivity without cell-free infectivity when inoculated to Vero or B95a cells. Furthermore, these virus particles were also neutralized by measles antibody. These results indicated that the supernatants contained cell-free infectious SSPE virus particles just like measles virus.

Because the Fr/V and Fr/B viruses were originally isolated with Vero and B95a cells, respectively, their persistent infections have been maintained in each of the original cell lines used for virus isolation. These two cell lines were infected with either the Fr/V or Fr/B pseudovirions (data not shown). The pseudovirions of the Fr/V virus formed clear plaques on both cells, producing 1.5 times more plaques on B95a cells than on Vero cells. In contrast, the Fr/B

Table 1 Comparison of sibling viruses (Fr/V and Fr/B viruses) of the Osaka-2 strain

	Fr/V	*Fr/B*	*Reference*
Cell type for isolation	Vero	B95a	1
Cytopathic effect	Syncytium	Syncytium	1
Production of cell-free virus	Very limited	Very limited	2
Hemadsorption	−	−	2
M protein synthesis	−	−	2
M mRNA	+	−	2
P-M bicistronic mRNA	+	+++	2
M gene sequence	Seven nucleotide differences		2
H gene sequence	Identical		3
F gene sequence	Three nucleotide differences (Two amino acid differences)		4

1. Ogura *et al*, 1997.
2. Seto *et al*, 1999.
3. Furukawa *et al*, 2001.
4. unpublished data.

pseudovirions did not detectably form plaques on Vero cells, although B95a cells showed high susceptibility to Fr/B pseudovirions. This relation of cell tropism between the Fr/V and Fr/B viruses resembled that between recent field isolates with lymphocytic cells such as B95a cells and laboratory strains such as the Edmonston strain long-term passaged with Vero cells, although the mechanism of the negative affinity of the Fr/B virus to Vero cells is not yet known.

Neurovirulence of the Fr/V and Fr/B viruses in hamsters

Pseudovirions of the Fr/V or Fr/B virus were inoculated intracerebrally in 3-week-old hamsters. As shown in Table 2, all the hamsters showed neurological symptoms, and almost all died even when inoculated with an Fr/V dose of 26 PFU and an Fr/B dose of 32 PFU. No significant difference was observed in latent periods between the Fr/V and Fr/B viruses, which were 4 to 13 days and 4 to 16 days, respectively. The survival times of the hamsters infected with the Fr/V virus were 1 to 6 days, except for two hamsters that recovered clinically, yet those of the hamsters infected with Fr/B were 3 to 17 days. Major symptoms included hypersensitivity, hypersalivation, involuntary movement of the neck or foreleg, myoclonus of the ears or foreleg, general convulsions, and so on. Six dying hamsters were sacrificed on days 3, 4, and 5 after onset of disease, and their brains were subjected to pathological examination. Although wider neural degeneration was observed in brains from the hamsters having the disease for a longer period, there were almost no inflammatory findings in any of the preparations (Figure 1A).

Large syncytium formation was found in the hippocampal region of only one of the hamsters examined (Figure 1B). In contrast, three hamsters inoculated intracerebrally with more than 10^5 PFU of the measles virus Masusako strain, the closest known relative measles virus of the SSPE virus Osaka-2 strain showed no signs of disease for 12 weeks (data not shown). Furthermore, inoculation of the Masusako strain prepared by the same cytochalasin D treatment of the infected cells as the pseudovirions of SSPE virus, injection of the same concentration of cytochalasin D itself or the same volume of culture medium of uninfected Vero or B95a cells induced no neurovirulence in hamsters (data not shown).

Molecular comparison of inoculated viruses and reisolated viruses from diseased hamster brains

By cocultivation with Vero or B95a cells, replicating viruses were reisolated from the whole brains of six dying hamsters on days 2 and 3 after onset of disease. Latent periods of these hamsters were 4 days for three Fr/V-inoculated hamsters and 6 to 9 days for three Fr/B-inoculated ones. Although reisolation was possible with both cell types from all six hamsters, isolation was easier with B95a than with Vero cells. In B95a cells, a large amount of syncytium formation appeared 2 to 3 days after cocultivation, yet this formation took a week on Vero cells. In order to clarify not only the neuropathogenicity of the inoculated viruses but also the differences between viruses reisolated with different cell types from the diseased brains, inoculated and reisolated viruses were analyzed genetically. Sequences were determined at the region containing characteristics of the two sibling viruses, the phosphoprotein (P) and M gene junction (Figure 2).

Two kinds of RNA preparations of the Fr/V and Fr/B viruses were sequenced: one (#1) is the RNA stock used in a study by Seto *et al* (1999), and the other (#2) is that from Vero and B95a cells infected with the pseudovirions, Fr/V and Fr/B, respectively, used in the present study. When comparing the M gene sequences between #1 and #2, three replacements, T to C, T to C, and A to C, at nucleotide

Table 2 Intracerebral inoculation of the Osaka-2 strain into hamsters at various doses

Virus	*Dose (PFU)*	*Onset of disease[1] (Survival time[2])*				*Incidence of disease[3]*
Fr/V	411	4 (3)	4 (3*)	4 (2)	4 (2)	4/4
	206	4 (5*)	4 (3)	4 (2)	4 (1)	4/4
	103	13 (92**)	5 (3)	5 (2)	4 (1)	4/4
	52	7 (4)	5 (5)	5 (4*)	4 (6)	4/4
	26	9 (164***)	8 (5)	7 (2)	5 (2)	4/4
Fr/B	511	6 (4)	6 (4*,****)	6 (3)	5 (5)	4/4
	256	8 (4)	7 (5)	6 (3)	4 (5*)	4/4
	128	16 (17)	16 (5)	12 (4)	9 (4)	4/4
	64	7 (5*)	7 (3)	6 (5)	6 (4)	4/4
	32	12 (6)	7 (7)	6 (5)	5 (9)	4/4

[1]Days from intracerebral inoculation to onset of disease.
[2]Days from onset of disease to death or sacrifice.
[3]Number of diseased hamsters/number of inoculated hamsters.
*Sacrificed at the terminal stage and subjected to pathological examination.
**Accidental death 105 days after inoculation (clinically recovered).
***Sacrificed 173 days after inoculation (clinically recovered).
****A syncytial giant cell in the hippocampus (HE stain).

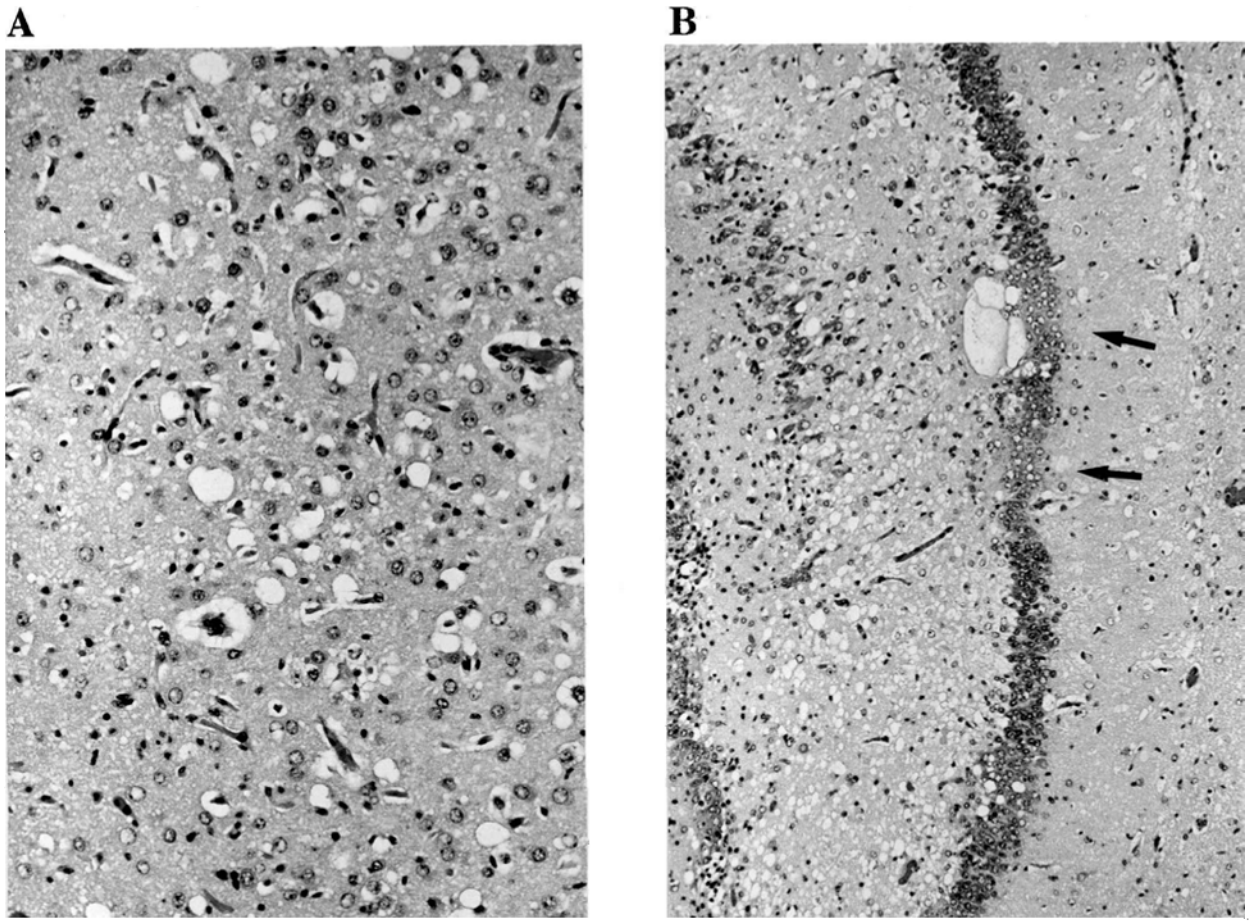

Figure 1 Histopathological analysis of infected hamster brains. Brains removed from sacrificed mice were fixed and brain sections were stained with hematoxylin and eosin. Diffuse spongiform degeneration without cell infiltration was observed in most hamsters (A), × 350: a hamster inoculated with 64 PFU of Fr/B with survival time of 5 days. Large syncytium formation (arrows) was found in the hippocampal region of only one of the Fr/B virus-inoculated hamsters (B), × 175: a hamster inoculated with 511 PFU of Fr/B with survival time of 4 days (see Table 2).

numbers 14, 45, and 59 of the M gene, respectively, were found in the Fr/V virus, and no replacements were detected in the Fr/B virus. In all the reisolated viruses from the Fr/V-inoculated hamster brains, only one difference, A to G at nucleotide number 80 of the M gene, was detected. There was also one difference, T to C at nucleotide number 29 of the M gene, in all the reisolated viruses from the Fr/B-inoculated hamster brains. No difference was found between cell types for virus reisolation and among the three hamsters inoculated with each of the sibling viruses. All the reisolated viruses maintained four of five nucleotides characteristic of the inoculated viruses, Fr/V and Fr/B, including nucleotide A at the 3′ end of the P gene and nucleotides C, C, and A of nucleotide numbers 29, 45, and 58 of the M gene, respectively, for the Fr/V virus, and nucleotide G at the 3′ end of the P gene and nucleotides T, G, and G of nucleotide numbers 45, 58, and 80 of the M gene, respectively, for the Fr/B virus. Only one nucleotide replacement, therefore, could be detected in 100 nucleotides of the reisolated viruses examined by RT-PCR. These data suggest that the inoculated viruses replicated and expressed neuropathogenicity in hamster brains.

Discussion

We studied the neuropathogenicity of the Fr/V and Fr/B viruses, two sibling viruses of the SSPE virus Osaka-2 strain, in hamsters, especially focusing on its relation to the cell types for virus isolation and the following passages and on the identity of the inoculated viruses with the growing viruses in hamster brains. Doses of only 26 PFU of the Fr/V virus and 32 PFU of the Fr/B virus induced encephalopathy in all the hamsters by intracerebral inoculation. The Osaka-2 strain showed extremely strong neurovirulence. The measles virus Masusako strain, which was isolated from a measles patient in Osaka in 1983, is the closest available progenitor virus of the Osaka-2 strain isolated from the brain of an SSPE patient who suffered from measles in 1984 in Osaka (Ogura *et al*, 1997; Ayata *et al*, 1998b). This Masusako strain, in contrast

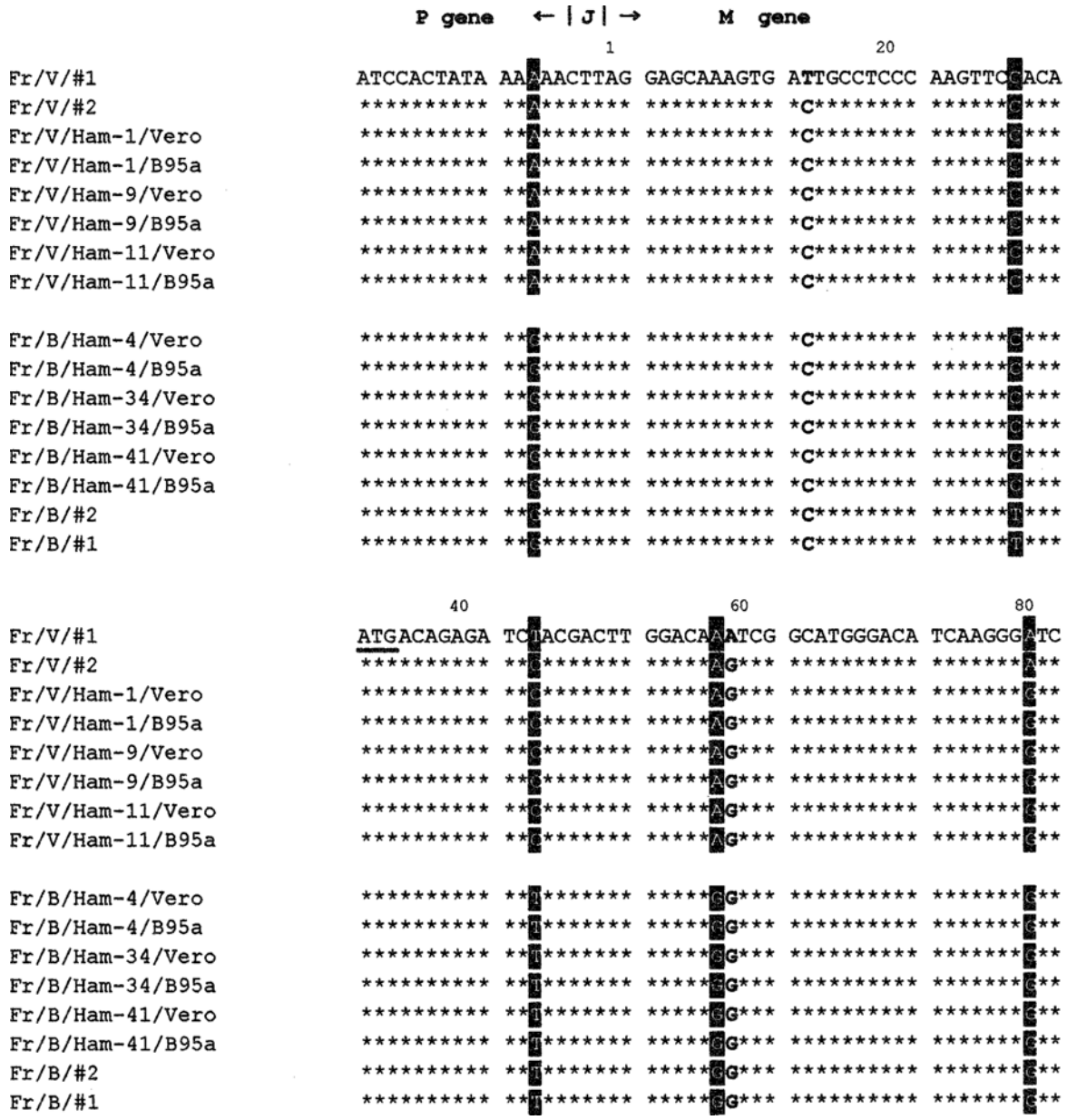

Figure 2 Alignments of the nucleotide sequences of the region (100 nucleotides) containing the P-M gene junction of the Fr/V and Fr/B viruses of Osaka-2 strain and reisolated viruses from diseased hamster brains. Two kinds of RNA preparations of the Fr/V and Fr/B viruses were sequenced after RT-PCR: one (#1) is the RNA stock used in a previous study by Seto *et al* (1999), and the other (#2) is that from Vero and B95a cells infected with the Fr/V and Fr/B pseudovirion preparations, respectively, used for intracerebral inoculation in the present study. Nucleotides are shown, excluding the region used for the design of primer sequences for PCR. Asterisks (*) represent the nucleotides identical to those of Fr/V #1. The translational start codon of the M gene was underlined. Five characteristic nucleotides that differ between the Fr/V and Fr/B viruses are expressed in reverse. | J | represents the trinucleotide of the P-M gene boundary. Fr/V/Ham-1/Vero represents virus reisolated with Vero cells from hamster number 1 inoculated with Fr/V virus.

to the result of the SSPE Osaka-2 strain, showed no neurovirulence in hamsters, even with an intracerebral inoculation of more than 10^5 PFU.

The Osaka-2 strain Fr/B virus was the first SSPE virus to be isolated by cocultivation with B95a cells (Ogura *et al*, 1997) and has been found to possess neurovirulence to rodents, similar to the Fr/V virus isolated with Vero cells. The symptoms of hamsters infected with the Fr/V and Fr/B viruses were found to be almost the same as those previously reported by Ohuchi *et al* (1981), Homma *et al* (1982), and Sugita *et al* (1984). Although both the Fr/V and Fr/B viruses have been found to be defective in M protein expression, monocistronic M mRNA has been detected in Fr/V-infected Vero cells but not in Fr/B-infected B95a cells (Seto *et al*, 1999). The present data, therefore, indicate that this transcriptional difference may not affect the neurovirulence to hamsters.

Half of the hamsters inoculated with the Fr/V virus became ill within 4 days of inoculation, yet only 1 of 20 hamsters inoculated with the Fr/B virus became ill within that time. In addition, 8 of 20 hamsters inoculated with the Fr/V virus died within 2 days after onset, whereas within 2 days there were no deaths in the Fr/B virus-inoculated hamsters. Based on these results, it seems that the Fr/V virus has a tendency to induce a more acute progression than the Fr/B virus. When compared to the results of Sugita *et al* (1984), neurovirulence of the Fr/V and Fr/B viruses appears to be similar to that of the Biken strain. The SSPE virus Yamagata-1 strain has been reported to induce slowly progressive disease by intracerebral inoculation in 3- to 6-week-old mice, most of which survive to more than 5 months (Homma *et al*, 1982). When we examined the neurovirulence of the Yamagata-1 strain in our pseudovirion-hamster system, one of five hamsters intracerebrally inoculated with 66 PFU showed encephalopathy 104 days after inoculation and was almost dying 28 days after onset, from whose brain the virus could be isolated (unpublished data). Although this strain is far higher fusogenic in Vero cells than the Osaka-2 strain Fr/V virus, the neurovirulence of the Yamagata-1 strain was extremely weaker rather than that of the Osaka-2 strain Fr/V virus. Further investigations should be needed in order to confirm the indication by Cathomen *et al* (1998). It would be also interesting to determine whether a stronger neurovirulent virus was selected in this case.

Based on the ability to form syncytia, a different cell tropism between Fr/V and Fr/B viruses for Vero and B95a cells was observed. Our recent results have revealed that the hemagglutinin (H) genes of the two sibling viruses are identical to each other (Furukawa *et al*, 2001), yet three different nucleotides have been found between fusion (F) genes of the two viruses and two of them resulted in amino acid replacements in the coding region (unpublished data). At present, there are no molecular data to account for the previous different cellular permissiveness, but the difference might not be involved in virus entry. Reisolation was carried out using Vero and B95a cells from the brains of six dying hamsters inoculated with the Fr/V and Fr/B viruses. The amount of syncytium focus in B95a cells was larger than that in Vero cells in all the hamsters. Although the inoculated Fr/B virus did not initially form syncytia on Vero cells, the Fr/B virus from diseased hamster brains could be reisolated with Vero cells. These reisolated Fr/B viruses needed longer periods to form clear plaques on Vero cells than on B95a cells. Sakaguchi *et al* (1985) have also reported that an aneurovirulent variant of the Biken strain obtained by serially passaging the strain more than 200 times through HEL (derived from human embryonic lung) cells reacquired neuropathogenicity after being passaged through human neural cells. The molecular background of these phenomena is quite intriguing, and our case is now under investigation. Because wild-type measles viruses can be isolated easily with B95a cells, but not with Vero cells (Kobune *et al*, 1990), a different receptor for the measles virus from CD46 has been suspected. Tatsuo *et al* (2000) have at last found a new receptor, SLAM (CDw150), for measles virus field isolates and have shown that field isolates only use SLAM for virus entry, yet laboratory strains use either SLAM or CD46. Because H genes cloned from the Osaka-1, -2, or -3 strain express no hemadsorption activity with African Green Monkey erythrocytes and positive hemadsorption activity is inhibited by anti-CD46 monoclonal antibody, it has been suggested that SSPE virus Osaka-1, -2, and -3 strains do not use CD46 for viral spreading (Furukawa *et al*, 2001). Because SSPE virus can be isolated more efficiently with Vero than with B95a cells (Ogura *et al*, 1997) and also can propagate fatally in hamster and mouse brains, some cell-surface molecules, other than SLAM and CD46, are possibly involved in SSPE virus entry.

To clarify the identity of the neurovirulent virus with inoculated virus, we genetically compared the inoculated and growing viruses in the brains, as such a confirmation has not been carried out in the past. We analyzed the 100-nucleotide sequences containing the P-M gene junction that are characteristic of the two sibling viruses. The sequences of the reisolated viruses from the brains were found to be almost identical to those of each inoculated virus, without differences among hamsters, further irrespective of the cell types for virus reisolation. These results suggest that the major population in the inoculum of the Osaka-2 strain replicates in hamster brains and expresses neuropathogenicity, which is due to some sequences being different from the possible progenitor virus, the Masusako strain. To clarify this intriguing phenomenon, in which altered regions of the SSPE virus genome are associated with neurovirulence, genetical homogeneity of the inoculated virus is thought to be essential. Therefore, investigations using recombinant measles viruses with SSPE virus genes by reverse genetics and site-directed mutagenesis of measles viruses are now in progress in our laboratory.

Materials and methods

Cells and viruses

Vero cells were cultured at 35°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% fetal calf serum and 4% newborn calf serum. B95a cells (Kobune *et al*, 1990) were cultured at 35°C in RPMI-1640 medium supplemented with 5% fetal calf serum. The SSPE virus Osaka-2 strain Fr/V and Fr/B viruses are sibling viruses isolated with Vero cells and B95a cells, respectively, from a biopsy specimen of the frontal lobe of brain of a patient with SSPE (Ogura *et al*, 1997). The nonproductive

persistent infection with each sibling virus, which was established immediately after the sibling viruses were isolated from the brain, has been maintained in a cell population. The persistently infected cells were cultured in the same manner as the parental cells and were passaged every 4 to 5 days. The measles virus Masusako strain was used as a possible progenitor virus, a measles virus that is the closest available field isolate to the SSPE virus Osaka-2 strain, was propagated in Vero cells (Ayata *et al*, 1998b).

Preparation of infectious pseudovirions from nonproductive SSPE virus-infected cultures
To prepare cell-free particles, pseudovirions, of the nonproductive Fr/V and Fr/B viruses for intracerebral inoculation into hamster brain, Fr/V-infected Vero cells or Fr/B-infected B95a cell cultures were replaced with serum-free DMEM containing 5 μg/ml of cytochalasin D (Sigma, St. Louis, MO, USA) when these cultures showed the maximum of syncytium formation mostly at 2- or 3-day cultivation. After incubation at 35°C for 30 min in a CO_2 incubator, the surface of the treated cultures was washed with the serum-free DMEM by gentle pipetting, and the supernatants were collected by centrifugation at 3000 rpm ($900 \times g$) for 15 min at 4°C and stocked at −85°C until use according to the method described by Ayata (1987).

Virus inoculation
Three-week-old female golden hamsters (SLC-Japan, Shizuoka, Japan) were used. After gently anesthetizing the hamsters with ether, 50 μl of pseudovirions of the Fr/V or Fr/B viruses were inoculated into the right hemisphere of the brain. Inoculum was prepared by a five-step, two-fold dilution in an experiment, and one dilution was inoculated into four hamsters per step. A possible progenitor measles virus of the Osaka-2 strain, the Masusako strain (2×10^5 PFU/50 μl) was inoculated into three hamsters in the same way as the pseudovirions. Infected hamsters were bred under specific-pathogen-free conditions in negative pressure isolators in infected animal rooms at the BSL-2 level in the Laboratory Animal Center of our Medical School. All animal experiments were performed according to the Guide for Animal Experimentation, Osaka City University.

Virus titration
Pseudovirions of the Osaka-2 strain were titrated by a plaque-formation method using the standard procedure for a measles virus titration.

Reisolation of virus from diseased hamster brains
When the inoculated hamsters were dying on days 2 and 3 after onset of disease, they were anesthetized with ether. Brains were aseptically removed for virus reisolation. The removed brains were cut into small pieces and treated with 0.25% trypsin. The single brain cells were cocultured with Vero or B95a cells. Reisolation was determined based on the appearance of syncytia. Infected cultures were passaged every 3 to 4 days if necessary.

Pathological examination
When the inoculated hamsters were dying on days 3, 4, and 5 after onset of disease, they were anesthetized with ether. Removed brains were fixed with 10% formalin (Wako Pure Chemicals, Osaka, Japan) and stained with hematoxylin and eosin according to the conventional method.

RNA extraction, reverse transcription (RT), polymerase chain reaction (PCR), and sequencing
The extraction of total cellular RNA from reisolated virus-infected cells and RT primed with a random primer (nonadeoxyribonucleotide mixture; Takara Shuzo, Otsu, Japan) were performed according to a previously described method (Ayata *et al*, 1998a). The region containing the P-M gene junction was amplified by PCR with a set of primers (MVPm1: 5′-CCTGCATCACGCAGTGTAATCC-3′ and MVMg5: 5′-CCAGTTTTTCATTGAGCCCTGC-3′) prepared on the basis of the published sequence for the Edmonston strain of measles virus. Amplification was carried out with Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) at 95°C for 2 min, 95°C for 1 min/50°C for 1 min/72°C for 3 min/40 cycles, and 72°C for 7 min. Amplified products were purified from a 0.8% agarose gel using a Qiaex II gel extraction kit (Qiagen GmbH, Hilden, Germany). PCR products were directly sequenced using a Thermo Sequenase II dye-terminator cycle sequencing kit (Amersham Pharmacia Biotech Ltd, Cleveland, OH, USA) and a model 373S sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA).

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