

**Neurovirulence in mice of soluble receptor-resistant (srr)
mutants of mouse hepatitis virus: intensive apoptosis
caused by less virulent srr mutant**

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Summary. Three soluble receptor-resistant (srr) mutants, srr7, srr11 and srr18, derived from a highly neurotropic mouse hepatitis virus (MHV) JHMV have a single amino acid mutation in the spike (S) protein. We examined using ICR mice whether the amino acids mutated in the mutants were involved in the neurovirulence. Srr7 showed apparently reduced neurovirulence relative to the wild-type (wt) JHMV in terms of the LD₅₀ and survival time, while the others showed slightly reduced virulence. In the brain and spinal cord, the growth of srr7 was more than 2 log₁₀ lower than that of the wt virus. Histopathologically, no significant difference was revealed between wt and srr7-infected mice on day 2 postinoculation (p.i.), with only scant inflammation and a minimum degree of neuropathological changes. The major difference was that apoptotic cells were frequently encountered in the srr7-infected mouse brain, but not in wt-infected mice on day 2 p.i. However, there was no difference between these viruses in the potential to induce apoptosis in cultured cells. The apoptosis in the brain did not appear to result from the direct viral attack, since apoptotic cells were found in the lesion where viral antigens were barely detected. The present study suggests that the amino acids mutated in the S protein of srr mutants, especially the amino acid at position 1114 mutated in srr7, influence the neurovirulence in mice.

Introduction

Mouse hepatitis virus (MHV), a member of *Coronaviridae*, causes various types of diseases in mice and rats, such as hepatitis, encephalomyelitis and enteritis [27, 35]. The disease pattern as well as pathogenicity of MHV is determined by a combination of the virus strains and host factors. The host factors include the species, age, and immune status [35], while a major viral factor is thought

to be the spike (S) protein which comprises the spikes protruding on the virion surface.

The S protein is a large membrane glycoprotein with a 180–200 kDa. It is cleaved into N terminal S1 and C terminal S2 subunits by host-derived protease [28]. These two subunits interact and constitute the petal-shaped spike with knob at the outer extremity and stem existing beneath it. The knob is supposedly formed of the S1 and the stem of the S2 [5]. In addition to the viral pathogenicity, S protein is responsible for a variety of important biological functions of MHV, e.g. the binding to the receptor protein, entry into susceptible cells as well as epitopes for neutralizing antibody and cytotoxic T cells [15, 30].

We isolated soluble receptor-resistant (srr) mutants from a highly neurotropic MHV JHMV strain to analyze the structure of the receptor-binding region in the S protein as well as the interaction between MHV receptor protein and viral S protein [26]. These srr mutants escaped from neutralization with soluble MHV receptor protein, CEACAM1^a [7, 36], which is expressed in most laboratory mouse strains highly susceptible to MHV [6, 38]. The three srr mutants we have isolated have a single mutation in either the S1 or S2 subunit [26]. These mutants grow in cultured cells as efficiently as the wt virus [22, 26], however, their fusogenicity is slightly alleviated relative to the wt virus, which is evident when cells were infected with a lower multiplicity of infection. This suggests that the single mutation in the S protein slightly changed their cytopathogenicity. Since the S protein is thought to be a key in determining the viral pathogenicity for animals [4, 10], we attempted, in the present study, to clarify whether the mutations observed in srr mutants affect the pathogenicity for mice.

Materials and methods

Viruses and cells

Neurotropic MHV JHMV cl-2 strain (wt) [32] and the srr mutants, srr7, srr11 and srr18, derived from cl-2 [26] were used. Srr7, srr11 and srr18 have a mutation at amino acid position 1114 (Leu → Phe), 65 (Leu → His) and 1163 (Cys → Phe), respectively. These viruses were propagated and plaque-assayed on DBT cells as described previously [33]. DBT [14] and 17cl-1 cells [29] were grown in Dulbecco's modified minimal essential medium (DMEM, Nissui, Tokyo) supplemented with 5% fetal calf serum (FCS, Gibco BRL, Grand Island, NY) and DMEM supplemented with 10% FCS, respectively.

Animals

Four-week-old male ICR mice, 21 to 25 g body weights, proved free from the infection of MHV and other murine pathogens, were purchased from Charles River Japan (Tsukuba, Ibaraki). They were inoculated intracerebrally (i.c.) with various doses of wt or srr mutants in 50 μ l phosphate buffered saline (PBS), pH 7.2, and observed for 9 days after infection. Mice were fed with commercial pellets and water *ad libitum* before and after infection.

Titration of infectious virus in organs

Mice were infected i.c. with 3×10^3 PFU of wt or srr mutants and killed at intervals after infection. Brains, spinal cords and livers were aseptically isolated from animals and kept at -80°C

until titration. These organs were homogenated with glass homogenizer in nine volume excess amounts of chilled PBS containing 200 µg/ml of kanamycin and the homogenates were centrifuged at 3,000 rpm for 10 min. The infectivity in the supernatants was measured by plaque assay using DBT cells as described previously [14, 33].

Histopathology

Mice inoculated i.c. with 3×10^3 PFU of wt or srr7 were killed at intervals after inoculation. Brains, spinal cords and livers were removed and fixed by 4% paraformaldehyde in 0.12 M phosphate buffer and processed for paraffin section as previously described [21]. Sections were stained with hematoxylin and eosin (H-E). Myelin was stained by Luxol-fast-blue (LFB). Viral antigens were visualized by polymerization of diaminobenzidine tetrahydrochloride (Dotite, Doujin, Kumamoto, Japan) after the ABC method [21] using rabbit anti-JHMV polyclonal antibodies, biotinylated anti-rabbit IgG (Amersham, Arlington Heights) and avidin-peroxydase complex (Zymed, San Francisco, USA). On the serial sections prepared for the detection of viral antigen, the apoptosis was examined by the TUNEL method using In Situ Cell Death Detection Kit (Boehringer Mannheim).

DNA laddering assay

Peritoneal macrophages isolated from ICR mice as well as 17cl-1 cells were used to examine the apoptosis caused by JHMV infection. The fragmentation of DNA of these cells following JHMV infection was examined as previously reported with slight modification [1, 13]. ICR mice were inoculated intraperitoneally with 2 ml sterile thioglycolate medium (Eiken, Tokyo, Japan, TGC in short). Peritoneal macrophages were collected from these mice 4 days after TGC inoculation and cultured at 37 °C for 1 day with DMEM supplemented with 5% FCS as previously reported [31]. Cultured macrophages as well as 17cl-1 cells were infected with wt or srr7 at a multiplicity of infection (MOI) of 1. After 20 h incubation, cells were washed with PBS, resuspended in 50 µl ice cold lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100 [pH 7.4]) and incubated on ice for 30 min. After centrifugation of the lysates at 16,000 rpm at 4 °C for 20 min, the supernatants were treated with 1 µl of 20 mg/ml RNase A for 1 h at 37 °C and then with 1 µl of 20 mg/ml proteinase K for 1 h at 37 °C. Samples were precipitated by isopropanol in the presence of 0.5 M NaCl. DNA samples collected by spinning at 15,000 rpm for 10 min were resuspended in 10 µl of TE and 5 µl of samples were electrophoresed through 2% agarose gel (GTG SeaKem) containing ethidium bromide.

Results

Neurovirulence of viruses as examined by survival time and LD₅₀

Mice were inoculated i.c. with 3×10^3 , 3×10^2 , 3×10 or 3 PFU of each virus and the mortality was checked daily for 9 days after infection. All mice inoculated with 3×10^3 PFU of wt virus and srr mutants died within 9 days postinfection (p.i.), although a difference in survival time was evident. Mice infected with the wt virus died significantly earlier than those infected with srr mutants, irrespective of the dose inoculated. After 3×10^2 and 3×10 PFU inoculation, all mice infected with wt or srr18 died, while some mice infected with srr11 or srr7 survived over the observation period. The LD₅₀ values calculated on day 9 p.i. were 3 PFU, 95 PFU, 5 PFU and 3 PFU for the wt virus, srr7, srr11 and srr18, respectively. These findings indicate that srr7 had the most reduced neurovirulence for ICR mice and srr11 and srr18 were slightly attenuated viruses in terms of survival time.

Clinically, mice infected with the wt virus developed central nervous system (CNS) signs, such as ataxic gait and hind-limb paralysis, from the second to third day p.i., when infected with 3×10^3 PFU of the virus. The CNS signs appeared delayed in mice inoculated with low titered wt virus and some of those survived over the observation period. Mice infected with *srr* mutants started displaying such CNS signs from day 5 or later and showed mild and prolonged CNS signs. Some *srr*-infected mice suffering CNS signs such as hind leg paralysis survived over the observation period.

Viral growth in the brain and spinal cords

We have compared the growth of wt and *srr7* in organs, because the most prominent difference in pathogenicity was observed between these two viruses. Since all mice died with a significantly different course of disease when inoculated with 3×10^3 PFU of wt and *srr7*, the following experiments were carried out under this condition. Virus titers in the brain, spinal cord and liver were measured at intervals after inoculation. As shown in Fig. 1, the wt virus titer in the brain on day 2 p.i. was about $2.5 \log_{10}$ PFU higher than the titers of *srr7*. Wt virus reached a peak titer, $6.2 \log_{10}$ PFU on day 3 p.i., while *srr7* failed to attain such a high titer even when it attained the peak on day 4 p.i. *Srr7* grew inefficiently in both brain and spinal cord relative to the wt virus growth. In addition, a similar difference in the virus titers was detected in the liver between the wt virus and *srr7*. These findings show that the low neurovirulence of *srr7* could result from reduced growth in the brain and spinal cords. High growth in the liver of wt virus (10^5 PFU/0.1 g) did not appear to be a major cause of death, since mice inoculated with the wt virus via the intraperitoneal route survived the infection despite similar virus titers in the liver.

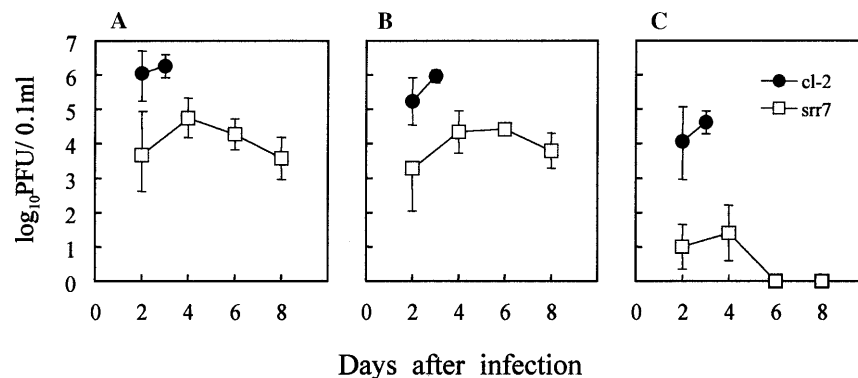


Fig. 1. Viral growth in the brain, spinal cord and liver. ICR mice were inoculated i.c. with 3×10^3 PFU of wt or *srr7* and viral growth in the brain (A), spinal cord (B) or liver (C) was measured. These organs were aseptically removed at intervals after inoculation and virus titers in the homogenized tissues were plaque assayed as described previously [33]. Each point shows the mean value and the vertical line extending above or below point indicates the standard deviation of three samples

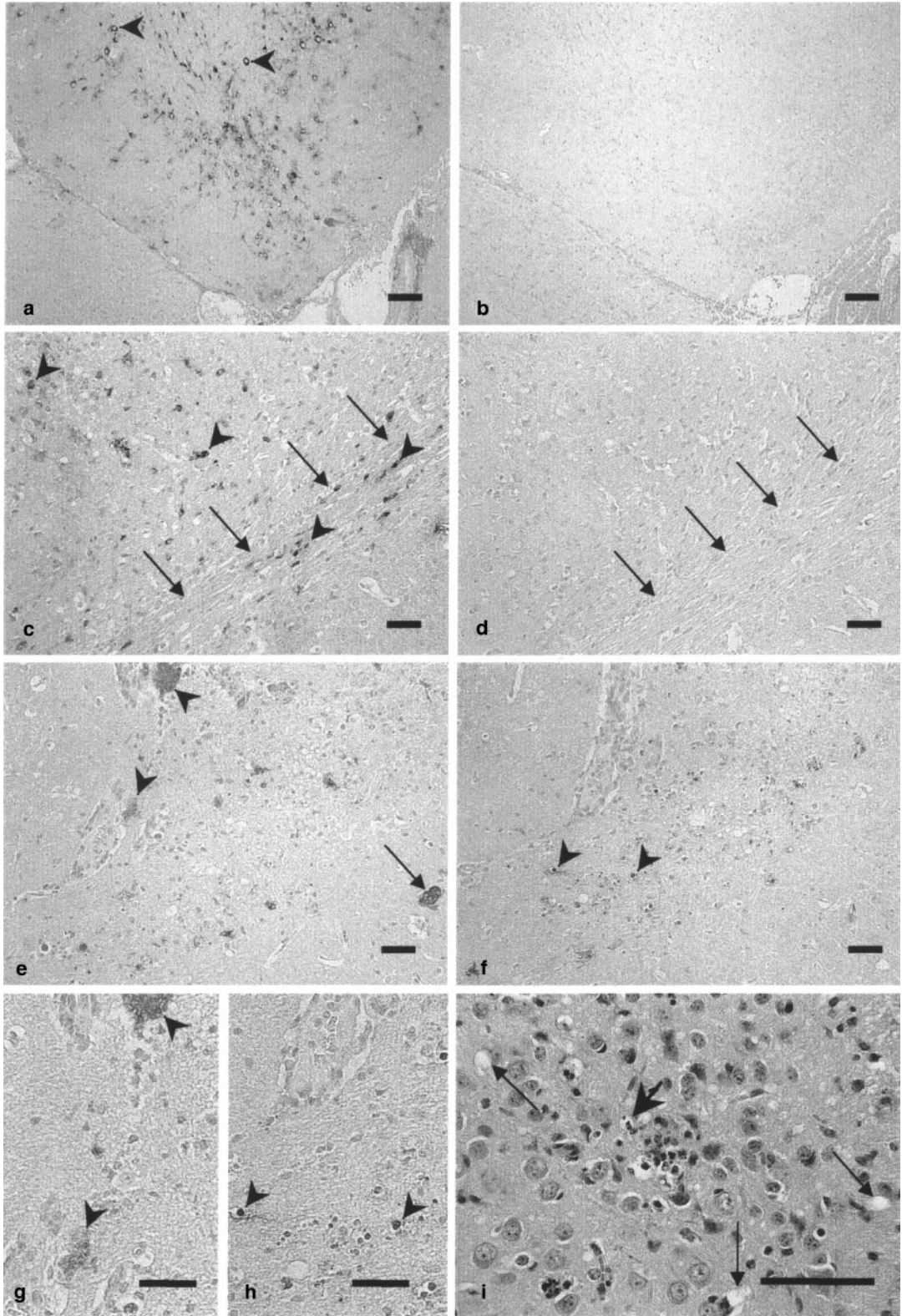
Histopathological examination

We defined the neuropathological examination of mice infected with wt and srr7. Mice inoculated with 3×10^3 PFU of wt or srr7 were killed at intervals after inoculation and brains and spinal cords were histopathologically examined. The comparison was mainly made on day 2 p.i., since most of the wt-infected mice died within 2 to 3 days after infection. On day 2 p.i., the neuropathological lesions of the infected mice remained at a minimal degree regardless of the virus type inoculated (Fig. 2). Mild infiltration in the meningeal space and small necrotic foci scattered in parenchyma (Fig. 2i) were found in the most of the infected animals. Despite the variation among individual mouse after infection with wt or srr7, the necrosis and inflammatory changes were slightly more intensive in the wt-infected mice than those infected with srr7. The immunohistochemical analysis revealed that viral antigens were distributed in large numbers of neurons in the cortex of wt-infected mice (Fig. 2a). Srr7 viral antigen was often detected in the white matter of the brain hemisphere as well as in the cortex (Fig. 2c).

A striking difference in pathology between wt and srr7 infection was found in the level of apoptosis in the parenchyme during the initial period of infection. Large numbers of apoptotic cells were observed in the brain parenchyme of srr7-infected mice (Figs. 2f and 3) on 2 day p.i., while only a few or no apoptotic cells were encountered in the same area of the wt-infected mice (Figs. 2b and 3). The localization of apoptotic cells did not coincide with that of viral antigen-positive cells. In the area where many antigen-positive cells were distributed, the apoptotic cells were relatively infrequently found (Figs. 2c and d). However, apoptotic cells were frequently encountered in the region adjacent to the cluster of antigen positive cells (Figs. 2e and f). Around this apoptosis rich region, clusters of small vacuolation were formed (Fig. 2i). Quantitative analysis showed a significant difference in the number of apoptotic cells in the brain between wt-infected mice and those infected with srr7 (Fig. 3). The low frequency of apoptosis in the wt-infected mice on day 2 did not result from the earlier event, since the occurrence of apoptosis was less frequent at 12 and 24 h in the wt virus-infected mice (Fig. 3).

Apoptosis induced by wt and srr7 in cultured cells

Since there was an apparent difference in the apoptosis caused by the wt virus and srr7 in the brain, we examined whether this difference was caused by their differential apoptotic potentials in cells infected with those viruses. Peritoneal macrophages isolated from ICR as previously reported [31] were used to compare the apoptotic activity of wt and srr7. These cells were infected with wt or srr7 at an MOI 1 and cell lysates prepared at 20 h p.i. were electrophoresed in 2% agarose gel to see the DNA fragmentation as a evidence of apoptosis. As shown in Fig. 4, DNA fragmentation was not significantly different between srr-infected macrophages and those infected with the wt virus. Since apoptosis was reported to occur in MHV infected 17cl-1 cells (1), we have also examined the apoptosis of those cells after wt and srr7 infection. Apoptosis of 17cl-1 cells infected with



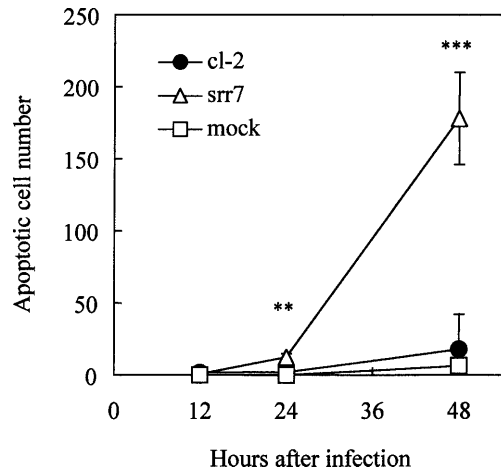


Fig. 3. Comparison of apoptotic cell number in the brain of wt- and srr7-infected mice. Three to six mice infected with either wt or srr7 and mock-infected mice were examined at 12, 24 and 48 h after infection. Apoptotic cells on the coronal cut surface of the brain at the level of infundibular recess of the third ventricle were counted after staining by the TUNEL method on the paraffin sections. Each point indicates the mean value of 3 to 6 samples and the vertical line extending above and/or below the point shows the standard deviation. **denotes statistically significant difference ($P < 0.05$ by student t test) between wt and srr7 at the time indicated

the wt virus was apparently greater than that of srr7-infected cells. This difference in the degree of apoptosis could be accounted for by higher growth of wt virus relative to srr7 in 17cl-1 cells (data not shown). These results showed that there was no significant difference in the apoptosis inducibility in cultured cells between wt and srr viruses, despite the difference in the degree of apoptosis in the brain of ICR mice. Since the envelope (E) protein has been reported to be responsible for apoptotic activity in cultured cells (1), we compared the E proteins of srr7

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Fig. 2. Immunostaining (a–h) and H-E staining (i) for paraffin sections prepared from mice 2 days after the infection with wt (a, b) or srr7 (c–i). On the serial sections prepared for the detection of viral antigen (a, c, e), apoptotic cells were ascertained by the TUNEL method (b), (d, f). After wt infection, viral antigen positive cells were spread in the brain cortex (a). The arrow head indicates infected neuron. In this area, no apoptotic cells were detected (b). Srr7 viral antigen positive cells (arrowhead) were often detected in the white matter (shown by arrows) of the brain hemisphere as well as in the cortex (c). In this viral antigen rich region, apoptotic cells were not prominent (d). (e) Clusters of viral antigen positive cells are formed in the meninx (arrowhead) and in the perivascular area (arrow) but the population of viral antigen positive cells in the brain parenchyma is much less than the area shown in (c). (f) On the serial sections in the area adjacent to (e), many apoptotic cells (arrowhead) were encountered in brain parenchyma. (g) Higher magnification of (e). (h) Higher magnification of (f). (i) Higher magnification of the area shown in (f) with H-E staining. Note a small necrotic focus which contains fragmented nuclei (short arrow). Many small vacuoles are beginning to form (long arrow). Bars indicate 70 μm

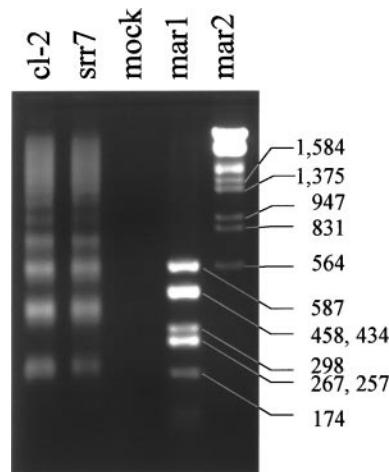


Fig. 4. Apoptosis of cultured peritoneal macrophages following infection with wt or *srr7*. Peritoneal macrophages harvested from ICR mice and cultured for 1 day were inoculated with wt or *srr7* at an MOI 1. DNA samples were prepared from those cells and also from mock-infected cells at 20 h after infection as described in Materials and methods. The DNA was electrophoresed in 2% agarose gel containing ethidium bromide. Marker 1 (*mar1*) indicates the pUC19 DNA fragments digested with Hae III and marker 2 (*mar2*) the λ DNA fragments digested with Hind III and EcoR I

with that of wt virus. Amino acid sequences deduced from nucleotide sequences showed no difference in their E proteins (data not shown). All these findings support the idea that the apoptosis caused in the mouse brains was not induced by the direct attack of viruses on the apoptotic cells. An alternative mechanism for the apoptosis in the mouse brain should be taken into account, such as through some cytokines produced as a result of virus infection in the brain.

Discussion

In the present study, we compared the neurovirulence of *srr* mutants and wt virus to address whether the amino acids mutated in *srr* S proteins were important for the neurovirulence of JHMV. Among 3 mutants, *srr7* showed the most markedly attenuated neurovirulence. Its attenuation was likely due to the reduced growth in the CNS relative to wt virus, although such difference was not observed in cultured cells [21, 26]. *Srr* mutants showed a slightly reduced fusogenicity in cultured cells relative to the wt virus with a low multiplicity of infection (unpublished data). Their reduced fusogenicity could account for the decreased viral spread in the mouse brain and eventually the low viral growth. Alternatively, the difference in growth between *srr7* and the wt virus in the mouse brain, but not in cultured cells, could be attributed to the difference of brain target cell sensitivity to these viruses. It is also possible that *srr7*, but not wt virus, induces some factors, such as interferon or cytokines, unfavorable for JHMV replication in the brain.

Srr7 has a mutation in the amino acid at position 1114 (Leu \rightarrow Phe) in the S2 subunit [26], suggesting that this amino acid is important for manifestation

of high neurovirulence. An MAb-escaped variant J2.2-v1 isolated from the DL strain of JHMV has a mutated amino acid at position 1114 (Leu → Phe) and this virus showed an attenuated neurovirulence [10, 34]. Although cl-2 and JHM-DL S proteins have two more different amino acids (position 298, Ser versus Thr; position 781, Val versus Gly), the mutation of the identical amino acid at position of 1114 resulted in a similar phenotypic change. This suggests the importance of this particular amino acid in the high neurovirulence of JHMV. However, we cannot exclude the possible involvement of the mutation outside the S gene of srr7. A virus with a mutation at position 1114 amino acid alone, which can be generated by a targeted RNA recombination [17, 20, 25], should be tested for its neurovirulence.

Another important biological difference between wt and srr7 was in apoptotic cell numbers in the brain. Apoptotic cells were more frequently found in srr7-infected mice than in wt-infected ones. The apoptosis did not appear to result from a direct viral attack, since the region rich in apoptotic cells was distinct from the region full of antigen-positive cells. Furthermore, the colocalization of viral antigen and TUNEL-positive nuclei was barely found. This feature was very similar to the JHMV J2.2-v1 infection reported by Wu and Perlman [37]. These findings suggest that apoptosis in the brain could be mediated by some cellular factors, such as cytokines. Interleukin-1 α (IL-1 α), IL-1 β , IL-6 and TNF- α were induced during viral clearance in MHV-4 (JHMV) infection [24]. These cytokines could be involved in the induction of apoptosis in srr7-infected mouse brain, in view of recent findings that those cytokines contribute to the neural cell apoptosis [2, 8, 9].

Apoptotic cell death could play an important role in host defense mechanism for eliminating infected cells. In support of this hypothesis, some viruses have acquired genes that block apoptosis, such as p35 and *iap* genes of baculovirus [3, 12]. Apoptosis may be an important antiviral mechanism in cells that can regenerate from surviving cells, however, it may result in functional disorders in the non-regenerative cells, such as neuronal cells. Virus-mediated apoptosis in the CNS generally results in the development of neurological diseases [11, 16, 18, 19, 23]. In these cases, the greater degree of apoptosis results in the more serious neurological diseases. In the present study, however, less virulent srr7 caused more serious apoptosis than did the highly virulent wt virus. It is apparent that the apoptosis by srr7 does not contribute to the development of neurological disorders. In contrast, the apoptosis could contribute to depress the neurovirulence. Little involvement of neurons in JHMV-mediated apoptosis could account for this situation. It is of interest to see how the greater apoptosis contributes to the suppression of virus neurovirulence.

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