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Localization of Neurovirulence Determinant for Rats on the S1 Subunit of Murine Coronavirus JHMV

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A cloned virus of murine coronavirus JHMV, cl-2, was shown to be highly neurovirulent for rats in comparison with other JHMV variants. We have isolated cl-2-derived variant viruses resistant to neutralization by monoclonal antibodies (MAbs) specific for the spike (S) protein of cl-2. The variants MM6 and MM13, selected by the MAbs specific for the JHMV S protein, were revealed to have a point mutation located within the N-terminal 100 amino acids (aa) of the S1 protein. The variants MM56, MM85, and MM78, selected by MAbs specific for the larger S protein of JHMV, were shown to have a deletion composed of about 150 aa located in the middle of the S1 subunit (MM56 and MM85) or one amino acid deletion, aspartic acid at number 543 from the N-terminus of the S1 (MM78). These five MAb-resistant variants were not different from cl-2 in growth pattern on cultured DBT cells. MM6 and MM13 were shown to be highly neurovirulent for 4-week-old Lewis rats, growing to high titers in the brain and causing as high an incidence of neurological disease and death as the parental cl-2. In contrast, MM56 and MM85 were nonneurovirulent for rats. They did not cause any central nervous system disorders nor did they multiply in the rat brains. MM78 showed intermediate neurovirulence as well as intermediate growth potential in the rat brain. However, there was no apparent difference in neurovirulence between the parental and the MAb-resistant variants for mice; all of these viruses showed high neurovirulence for mice. These results suggest that the domain composed of about 150 aa in the middle of the S1 is critical for high neurovirulence of JHMV for rats. Furthermore, it is suggested that the neurovirulence of cl-2 for mice is controlled by a different viral factor.

INTRODUCTION

Mouse hepatitis virus (MHV) is a member of the coronavirus family which are enveloped viruses with a single-stranded, positive-sense RNA of 28 to 32 kb (Boursnell *et al.*, 1987; Lee *et al.*, 1991; Spaan *et al.*, 1988). MHV includes various strains showing different organ tropisms as well as different pathogenicities. A neurotropic MHV strain, JHMV, causes acute and subacute encephalomyelitis in mice (Wege *et al.*, 1982). Some of the variant viruses isolated from JHMV are highly neurovirulent for rats as well (Matsubara *et al.*, 1991; Nagashima *et al.*, 1978).

The spike (S) protein of the coronavirus, a glycoprotein of 150 to 200 kDa, constitutes the projections on the virion surface. In most coronaviruses, the S protein is cleaved into S1 and S2 subunits derived from the N-terminal and C-terminal halves of the S protein (Siddell *et al.*, 1983; Spaan *et al.*, 1988; Sturman and Holmes, 1984). The S protein of MHV is of great interest since it retains a variety of the important biological functions that the virus shows (Collins *et al.*, 1982; Holmes *et al.*, 1981; Spaan *et al.*, 1988). It is involved in the binding to the MHV-specific receptors (Collins *et al.*, 1982; Holmes *et al.*, 1981), and the N-terminal region of the S1 has recently been suggested to interact with the receptor (Kubo *et al.*, 1994). The fusion of cultured cells infected with MHV is caused by the S protein (Spaan *et al.*, 1988). Even uncleaved S protein has active fusion activity (Stauber *et al.*, 1993; Taguchi, 1993). The S protein is the major target of the neutralizing antibodies induced in mice after infection with MHV. It also elicits cytotoxic T cells (Kyuwa and Stohlman, 1990). Furthermore, the S protein is suggested to be implicated in the pathogenicity of the virus.

The S protein is suggested to be a major determinant of the neurovirulence of JHMV for mice. The variants that escaped from neutralization of JHMV by the monoclonal antibodies (MAbs) specific for the S protein showed altered neurovirulence for mice (Dalziel *et al.*, 1986; Fazakerley *et al.*, 1992; Fleming *et al.*, 1986, 1987; Wege *et al.*, 1988). The nucleotide sequencing analyses of such variants have identified the region on the S protein critical for the high neurovirulence for mice (Fazakerley *et al.*, 1992; Wang *et al.*, 1992), although it is to be determined whether a single or multiple sites of the S gene are implicated in the neurovirulence (Wang *et al.*, 1992). However, little is known of the viral factor influencing the neurovirulence of JHMV for rats.

The S protein is speculated to be involved in neurovirulence for rats. Comparison of neurovirulence of JHMV variants suggested that the variants with a small S protein were nonneurovirulent, while those with a large S protein were neurovirulent (Matsubara *et al.*, 1991). These variants have variable differences in their S genes (Banner *et al.*, 1990; Parker *et al.*, 1989; Schmidt *et al.*,

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1987; Taguchi *et al.,* 1992) and possibly in the other genes as well. In order to analyze the role of the S protein in neurovirulence for rats, the viruses whose variations are restricted in the S gene should be compared. Thus, we have obtained the variant viruses resistant to neutralization by MAbs specific for the S protein (MAb-resistant variants) since it has been shown in several virus systems that the MAb-resistant variants usually arise by single point mutation (Evans *et al.,* 1983; Knossow *et al.,* 1984; Seif *et al.,* 1985). We have tested the neurovirulence of those variants for rats and found the variants with altered neurovirulence. The sequence analyses of the S1 genes have revealed that the domain of the S1 protein located in the middle of the S1, which is frequently found to be deleted during the passage of the virus with larger S protein (Gallagher *et al.,* 1990; Parker *et al.,* 1989), is actually involved in the neurovirulence of JHMV for rats.

MATERIALS AND METHODS

Viruses and cells

A highly neurovirulent JHMV variant, cl-2 (Taguchi *et al.,* 1985), was used as a parental virus to isolate MAb-resistant variant viruses. cl-2 virus was plaque purified three times before isolating the MAb-resistant viruses. Nonneurovirulent sp-4 virus (Taguchi and Fleming, 1989) was also used in some experiments. The viruses, parental cl-2, sp-4, and cl-2-derived MAb-resistant variants, were propagated and assayed on DBT cells as previously reported (Taguchi *et al.,* 1980). DBT cells were grown in Dulbecco's modified minimal essential medium (DMEM; Nissui, Tokyo) supplemented with 7% calf serum (CS; Gibco) and 10% tryptose phosphate broth (TPB; Difco, Detroit).

Growth of cl-2 and its MAb-resistant variants in cultured DBT cells

DBT cells prepared in 12-well plates (Coster, Cambridge) were infected with cl-2 or other MAb-resistant variants at a multiplicity of 1 PFU/cell and incubated at 37° for 1 hr. After three washes with DMEM, infected DBT cells were fed with DMEM containing 7% CS and 10% TPB. At intervals after infection, the culture fluids as well as cells were collected. Each sample was freeze-thawed three times to disrupt cells and infectious virus titers in samples were determined by plaque assay as previously reported (Taguchi *et al.,* 1980).

MAbs used for selecting viruses

For the selection of MAb-resistant variant viruses, we used the MAbs specific for the S1 subunit of the cl-2 virus prepared in our laboratory as described previously (Kubo *et al.,* 1993). The MAbs we prepared were reactive to most of the MHV strains examined (group A), reactive specifically to JHMV variants (group B), or reactive only to JHMV with the larger S protein (group C). Ascitic fluids of MAbs classified into groups B and C with viral neutralization activity were prepared by injecting $1-2 \times 10^6$ hybridoma cells into the peritoneal cavity of BALB/c mice and used to select MAb-resistant variant viruses.

Isolation of MAb-resistant variant viruses

cl-2 virus, 10^6 PFU in 100 μl of DMEM containing 10% TPB, was mixed with 5–10 μl of each MAb specific for the S1 subunit of the cl-2 S protein (Kubo *et al.,* 1993) and the mixture was incubated at room temperature for 45 to 60 min. The mixture was then inoculated onto the DBT cell monolayer prepared in six-well plates (Falcon) and cells were incubated at 37° for 1 hr for virus adsorption. One to 2 days after overlay of DMEM containing 2% CS, 10% TPB, and 1% Bacto agar (Difco), plaques were isolated and each MAb-resistant variant was further plaque purified three times using DBT cells. The isolated virus clones were finally confirmed to be resistant to MAbs used for selection. MAb-resistant variants were propagated in large scale, divided into small aliquots, and stored at −80° until use.

Isolation and sequencing of cDNA to the S1 genes of MAb-resistant variants

cl-2 and its MAb-resistant variant viruses were inoculated onto DBT cells prepared in 60-mm petri dishes (Falcon) at the m.o.i. of 1 to 5 and total RNA was isolated from cells when fusion of infected cells covered about 50% of cells by the method described by Chirgwin *et al.* (1979). The cDNA was then synthesized from the RNA with an oligonucleotide, SN-2381 (5′-TATCATTGACTA-ACATCGGC-3′), complementary to the nucleotide sequence of the cl-2 S gene as a primer by M-MLV reverse transcriptase (BRL, Gaithersburg, MD). The cDNA was amplified by polymerase chain reaction (PCR) (Yamada *et al.,* 1993) with different pairs of oligonucleotides prepared according to the published sequences of the S1 gene of cl-2 (Taguchi *et al.,* 1992). The amplified cDNA fragments, 600 to 1300 bp, were electrophoresed in an agarose gel and purified by Prep-A gene (Bio-Rad, Richmond, CA). The purified DNA fragments were directly sequenced by the method described by Winship (1989) by dideoxy chain termination using a sequencing kit (USB, Cleveland, OH).

Animal experiments

For animal experiments, 4-week-old male Lewis rats, 4- or 6-week-old male ICR mice, and 6-week-old male BALB/c mice were used. They were all purchased from Charles River Japan (CRJ, Atsugi, Kanagawa) and checked to be free from infection with MHV and other murine pathogens by enzyme-linked immunosorbent assay. After anesthesia by ether, Lewis rats were inoculated intracerebrally with 50 μl of MAb-resistant viruses in various doses 1 to 2 days after arrival. Infected rats were freely fed and clinically observed for 4 to 5 weeks

after infection. In some experiments, virus titers in the brains were assayed. The infected Lewis rats were killed by chloroform and brains were asceptically collected. The brains were homogenized in chilled phosphate-buffered saline, pH 7.2 (10% wt/vol), and centrifuged at 3000 rpm for 10 min at 4°. The supernatants were serially diluted and infectious virus titers were determined by plaque assay (Taguchi *et al.*, 1980). ICR mice as well as BALB/c mice were inoculated intracerebrally with various doses of parental cl-2 and MAb-resistant viruses at 1 day after arrival and observed clinically for 10 days after infection.

RESULTS

Sequence analysis of the S1 genes of MAb-resistant viruses

We have obtained the variant viruses from highly neurovirulent cl-2 virus after neutralization with the S1-specific MAbs. Fifteen different MAbs against the S1 subunit of cl-2 virus were classified into three distinctive groups on the basis of reactivities to various MHV strains (Kubo *et al.*, 1993). MAbs classified into group B, which is reactive specifically to JHMV variants, and those classified into group C, reactive only with JHMV containing the larger S protein, showed the viral neutralization activity (Kubo *et al.*, 1993). The cl-2 virus, 1×10^6 PFU/0.1 ml, was mixed with one of these MAbs and incubated at room temperature for 45 min and the surviving viruses were plaque purified. The plaque-purified viruses were confirmed again to be resistant to each MAb used to select them. Variants obtained after neutralization by MAbs 6 and 13 classified in group B were designated MM6 and MM13, respectively. MM78, MM56, and MM85 were isolated after neutralization of cl-2 with three MAbs classified in group C, MAbs 78, 56, and 85. We have isolated the RNA from DBT cells infected with each of these viruses and prepared cDNA to the S1 gene (nucleotides 1 to 2307 of the cl-2 S gene) by reverse transcription PCR. The DNA fragments were then directly sequenced by the method described by Winship (1989). The amino acid (aa) sequence of a newly isolated cDNA of cl-2 S gene (R-cl-2-S) was revealed to be very similar to that of MHV-4, showing only one aa difference at position 742 (Pro in R-cl-2-S and Thr in MHV-4) from the N-terminus of the S protein. Direct sequencing analysis of the DNA fragments amplified using RNA isolated from cl-2-infected DBT cells showed that the R-cl-2-S gene was a major genotype of the cl-2 S protein. The aa sequences of MAb-resistant variants deduced from nucleotide sequences were compared with that of parental JHMV cl-2 (R-cl-2-S), as shown in Table 1 and Fig. 1. MM6 and MM13 were revealed to have point mutations at aa 83 (Gly to Glu) and 26 (Asn to His) from the N-terminus of the S protein, respectively. Other than these positions in MM6 and MM13 there was no aa change in the S1 subunit. These results are in good agreement with our previous finding that the MAbs classified in group B recognize the epitopes located in the N-terminal 330 aa of the S1 subunit (Kubo *et al.*, 1994). Variants selected with MAbs 56 and 85 classified into group C were shown to contain a deletion of a stretch composed of 154 and 152 aa, respectively, as shown in Table 1 and Fig. 1. In addition, these variants obtained 1 new aa. MM56 obtained a new aa, isoleucine, from a codon ATT which was formed by the first nucleotide of Arg (A) at position 449 and the last two nucleotides of Phe (TT) at position 602. MM85 obtained a serine from a codon TCA which was formed by the first two nucleotides of serine (TC) at position 144 and the last nucleotide of Val (A) at position 195. MM78, selected by MAb 78 in group C, was different from the other two variants selected by MAbs in group C. MM78 contained only one aa deletion at position 543, aspartic acid.

Comparison of the growth of MAb-resistant viruses in cultured DBT cells

DBT cells were infected with parental cl-2 as well as its MAb-resistant variants at a multiplicity of 1 PFU/cell and the growth of these viruses and cytopathic changes caused by these viruses were compared. As shown in Fig. 2, there was no apparent difference in the growth of these viruses in DBT cells. Under the above condition, virus titers of all of these viruses reached a plateau at 12 hr postinoculation, approximately 1×10^6 PFU/0.1 ml. The cytopathic changes caused by these viruses were not distinguished. As eary as 6 hr postinoculation, polykaryocytes started being formed in DBT cells infected with all of these variants and almost all of the cells in dishes were fused at 12 hr. The size of plaques produced by these MAb-resistant variants was not different from that of cl-2 (data not shown).

Neurovirulence of MAb-resistant viruses for rats

To examine the neurovirulence of these variant viruses selected with various MAbs, we have inoculated 10^5 PFU of variant viruses into the brain of 4- to 5-week-old Lewis rats and observed clinically for 4 to 5 weeks after inoculation. As shown in Table 2A, MM6 and MM13, with a point mutation within 100 aa from the N-terminus, were demonstrated to be highly neurovirulent for rats as was their parental cl-2. About 80% of rats infected with these viruses showed clinical central nervous system (CNS) disorders such as ataxic gait and hind-limb paralysis, as reported previously (Matsubara *et al.*, 1991), within 12 days postinoculation (p.i.) and most of these succumbed within 15 days. However, MM56 and MM85 showed completely different neurovirulence. The rats inoculated with these viruses showed no clinical symptoms and did not die during the observation period. MM78 with one amino acid deletion was revealed to be of low virulence in comparison with the parental cl-2, MM6, and MM13; about 30% of rats inoculated showed the CNS disorders and

TABLE 1

Nucleotide Changes and Deletions in the S Gene of MAb-Resistant Variants

Variant	Nucleotide change[a]	Amino acid change[b]	Nucleotide deletions[c]	Amino acid deletions[d]
MM6	248: GGC → GAC	83: Gly → Glu		
MM13	76: AAT → CAT	26: Asn → His		
MM78			1627–1629	543 (1 aa)
MM56			1346–1804	449–602 (154 aa) +Ile
MM85			1332–1784	444–595 (152 aa) + Ser

[a] Nucleotide number: change.
[b] Amino acid number: change.
[c] Numbers of deleted nucleotide.
[d] Numbers of deleted amino acid (total number of deleted aa).

less than 10% of inoculated rats died. We further examined the responses of rats inoculated with serially diluted MAb-resistant variants with different neurovirulence for rats. As shown in Table 2B, rats inoculated with more than 10^3 PFU of parental cl-2 and MM13 showed responses similar to those of rats who were inoculated with 10^5 PFU. Mortality and time to death were not dependent upon doses of the virus inoculated. From these results, the LD_{50}'s of cl-2 and MM13 for rats were calculated to be less than 10^3 PFU, while those of MM78 and MM85 were more than 10^5 PFU. We then examined whether such neurovirulence correlated with the viral replication in the brain of rats. All of the above variants, at 10^5 PFU, were intracerebrally inoculated into Lewis rats and the rats were sacrificed on 6th day p.i., when the virus titers in the brains of cl-2-infected rats were revealed to plateau in our previous paper (Matsubara *et al.*, 1991). The virus titers in the brain were examined by plaque assay. As shown in Table 3, highly virulent cl-2, MM6, and MM13 were revealed to grow well (10^3 to 10^4 PFU/g) compared with the avirulent MM56 or MM85 ($<2.5 \times 10$ to 5×10 PFU/g). MM78, with intermediate virulence, showed titers in the brain intermediate (10^2 to 10^3 PFU/g) between high- and nonneurovirulent viruses. These results clearly showed that the neurovirulence of cl-2 and its variants correlated well with the growth potential in the brain of rats.

Neurovirulence of MAb-resistant viruses for mice

To see the neurovirulence of these MAb-resistant viruses for mice, we have inoculated various doses into the brains of 4-week-old ICR mice. As shown in Table 4A, most of the mice inoculated with 2 PFU of each variant died with neurologic disorders within 2 to 6 days p.i.; the LD_{50}'s of these variants were calculated to be more or less 1 PFU. Since the LD_{50}'s of MHV-4 and JHM were reported to be less than 0.1 PFU (Fazakerley *et al.*,

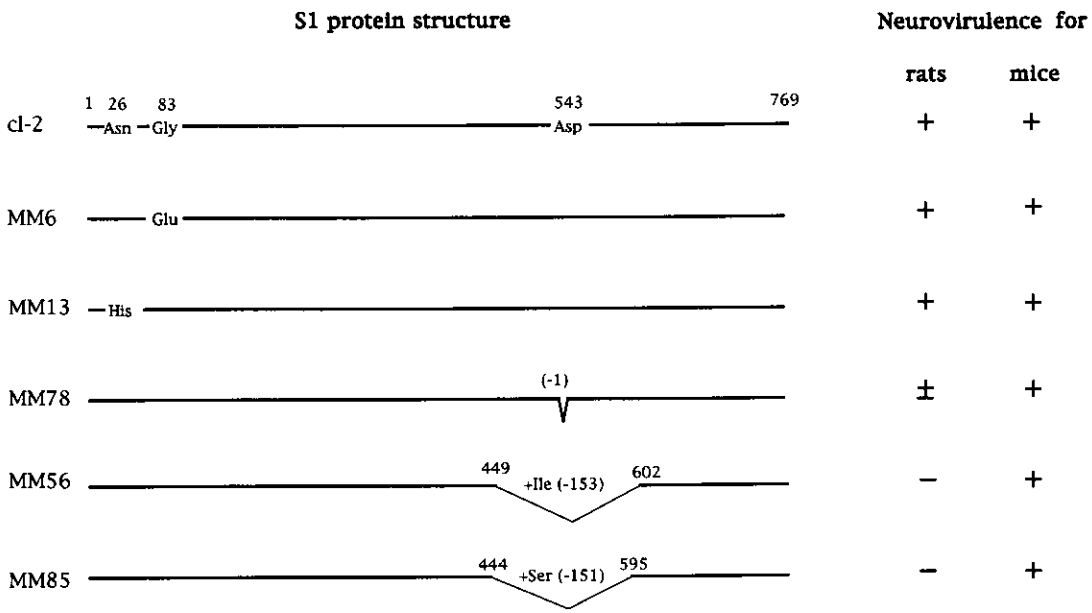

FIG. 1. Comparison of the S1 proteins and neurovirulence for rats and mice of parental cl-2 virus and its MAb-resistant variant viruses. MM6 and MM13 had a point mutation at aa 83 (Gly → Glu) and 26 (Asn → His), respectively. MM78 had a deletion of aa 543 (Asp). MM56 and MM85 had 153 and 151 aa deletions from aa 449 to 602 and from 444 to 595, respectively.

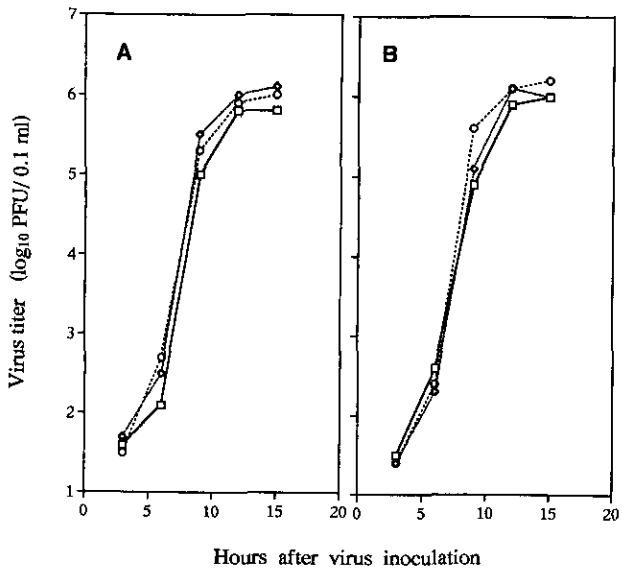

FIG. 2. Growth of cl-2 and its MAb-resistant variants in DBT cells. DBT cells were infected with cl-2 (□), MM6 (◇), or MM13 (○) shown in A or with MM56 (□), MM78 (◇), or MM85 (○) shown in B and their virus titers were examined at 3, 6, 9, 12, and 15 hr postinoculation.

1992; Wege *et al.*, 1988), we have calculated precisely the LD_{50}'s of three viruses with different neurovirulence for rats using 6-week-old ICR mice. As shown in Table 4B, the LD_{50}'s of cl-2, MM6, and MM85 were found to be 0.6, 1.2, and 0.3 PFU, respectively. These values were not remarkably different from each other and also not from the LD_{50} of JHMV obtained using ICR mice from the same breeder in 1981 (Hirano *et al.*, 1981). Thus, all of these data suggested that there was no apparent difference in neurovirulence for mice among these MAb-resistant variants.

This observation did not agree with the results obtained by Fazakerley *et al.* (1992) and by Wang *et al.* (1992), which showed that the MAb-resistant variant viruses V5A13.1 and 2.2/7.2-V-2 isolated from MHV-4 and JHM-DL, respectively, were neuroattenuated for mice compared with their parental viruses. The S genes of MHV-4 and JHM-DL are similar to that of cl-2 (Banner *et al.*, 1990; Parker *et al.*, 1989), and V5A13.1 and 2.2/7.2-V-2 have a deletion in the middle of S1, like MM56 and MM85. The major difference observed between our system and others was the mouse strain used for infection. We then examined the neurovirulence of our MAb-resistant viruses for BALB/c mice that were used by Fazakerley *et al.* (1992). We inoculated 10^3 PFU of cl-2, MM6, MM85, and MM78, as well as sp-4, showing different neurovirulence for rats, and clinically observed these mice for 1 week. As shown in Table 5, there was no remarkable difference in the mortality and time to death of mice inoculated with each of these variants, showing clearly that there was no difference in neurovirulence even for BALB/c mice among our MAb-resistant variants. The difference in neurovirulence of JHMV observed in different laboratories may result from different point mutations in the S proteins of MAb-resistant viruses isolated

TABLE 2

Neurovirulence of MAb-Resistant Variants for Rats

	Virus inoculated	Group of MAb for selection	Virus titer[a] inoculated	Mortality[b]	Time to death[c] (mean value)
A	cl-2	—	2×10^5	8/10/12	7–17 (11.3)
	MM6	B	2×10^5	10/12/14	9–17 (12.4)
	MM13	B	2×10^5	6/9/11	7–12 (9.0)
	MM78	C	2×10^5	1/4/12	10 (10)
	MM56	C	2×10^5	0/0/17	
	MM85	C	2×10^5	0/0/17	
B	cl-2	—	1×10^5	3/6/6	7–9 (8.0)
			1×10^4	3/4/6	8–12 (9.6)
			1×10^3	3/5/6	10–12 (11.0)
	MM13	B	1×10^5	2/5/6	6–8 (7.0)
			1×10^4	5/6/6	6–11 (8.4)
			1×10^3	3/6/6	9–12 (10.6)
	MM78	B	1×10^5	1/1/6	12
			1×10^4	1/1/6	12
	MM85	B	1×10^5	0/0/6	
			1×10^4	0/0/6	

Note. Four-week-old Lewis rats were inoculated intracerebrally with various doses of variants and clinically observed for 4 weeks after inoculation.

[a] PFU/50 μl.

[b] No. dead/No. with clinical symptom/No. inoculated.

[c] In days.

TABLE 3

Virus Titer in the Brain of Rats Inoculated with MAb-Resistant Variants

Virus inoculated	Rat	Virus titer in the brain[a]
cl-2	1	1.5×10^4
	2	1.1×10^4
	3	8.2×10^3
	4	4.3×10^3
MM6	1	6.0×10^3
	2	1.2×10^4
	3	5.2×10^3
MM13	1	1.3×10^4
	2	1.0×10^4
	3	1.1×10^3
MM78	1	3.2×10^3
	2	8.0×10^2
	3	5.2×10^2
MM56	1	2.5×10
	2	5.0×10
	3	$<2.5 \times 10$
MM85	1	$<2.5 \times 10$
	2	$<2.5 \times 10$
	3	$<2.5 \times 10$

Note. Lewis rats were inoculated intracerebrally with 1×10^5 PFU of variants and virus titers in the brain on Day 6 postinoculation were examined.

[a] PFU/g.

in different laboratories or it may be due to unidentified viral genetic differences in the genes outside the S gene.

DISCUSSION

There are only a few studies on the viral factors influencing the neurovirulence of JHMV for rats, although such factors for mice have been well documented (Dalziel *et al.*, 1986; Fazakerley *et al.*, 1992; Fleming *et al.*, 1986, 1987; Wege *et al.*, 1988). The variants with the larger S protein, isolated from the brains of infected rats, and those from the astrocyte culture infected with JHMV with the small S protein (Taguchi *et al.*, 1985, 1986), were reported to be highly neurovirulent for rats, although the parental JHMV with the small S protein was aneurovirulent (Matsubara *et al.*, 1991). It was also reported by Morris *et al.* (1989) that the variant At11f cord virus isolated from spinal cord of diseased rat contained a smaller S protein and showed low virulence for suckling rats compared with its parental JHMV. These variants with the small S protein were revealed to have 140- to 150-aa deletions in the hypervariable region in the S1 protein (Monica *et al.*, 1991; Schmidt *et al.*, 1987; Taguchi *et al.*, 1992). These observations may suggest that the larger S proteins of variants play a critical role for the high neurovirulence of JHMV for rats. However, the failure of these experiments to locate the neurovirulence determinant in the S gene may be due to the possible variations in the genome outside the S gene. The present study using MAb-resistant variants with the deletion in the S protein, whose genomes are conceivably very similar except for mutations selected by MAbs, as shown in other MAb-resistant variant viruses (Evans *et al.*, 1983; Knossow *et al.*, 1984; Seif *et al.*, 1985), revealed that a region composed of about 150 aa in S1 is critically involved in the high neurovirulence for rats. This is the first case to show directly that the S protein of JHMV is implicated in neurovirulence for rats.

It was shown in the present study that parental JHMV cl-2 and its MAb-resistant variants were not different regarding neurovirulence for mice; they were all highly neurovirulent for ICR and BALB/c mice. This observation is not in accordance with the results reported by Fazakerley *et al.* (1992) and by Wang *et al.* (1992). They showed that

TABLE 4

Neurovirulence of MAb-Resistant Variants for Mice

	Virus inoculated	Virus titer inoculated[a]	Mortality (time to death)[b]	LD_{50}[c]
A	cl-2	2×10	4/4 (3–4)	
		2	3/4 (2–4)	
	sp-4	2×10	4/4 (3–5)	
		2	4/4 (4–5)	
	MM6	2×10	4/4 (2–5)	
		2	4/4 (3)	
	MM13	2×10	4/4 (4–5)	
		2	4/4 (2–4)	
	MM78	2×10	4/4 (3–4)	
		2	3/4 (2–5)	
	MM56	2×10	4/4 (2–5)	
		2	4/4 (5–6)	
	MM85	2×10	4/4 (3–5)	
		2	4/4 (4–6)	
B	cl-2	2×10	4/4 (4–5)	
		2	2/4 (5)	0.6
		0.2	2/4 (5)	
		0.02	0/4	
	MM6	2×10	4/4 (5)	
		2	2/4 (5)	1.2
		0.2	1/4 (6)	
		0.02	0/4	
	MM85	2×10	4/4 (5–6)	
		2	3/4 (5–6)	0.3
		0.2	1/4 (5)	
		0.02	0/4	

Note. Four-week-old (A) and 6-week-old (B) ICR mice were inoculated intracerebrally with various doses of each virus and clinically observed for 10 days.

[a] PFU/50 μl.

[b] No. dead/No. inoculated (survival time in days).

[c] LD_{50} is expressed in PFU.

TABLE 5

Neurovirulence of MAb-Resistant Variants for BALB/c Mice

Virus inoculated	Mortality[a]	Time to death (mean value)[b]
cl-2	8/8	2–3 (2.9)
MM13	8/8	3–4 (3.4)
MM78	8/8	2–4 (2.8)
MM85	9/9	3–5 (3.9)
sp-4	9/9	4–5 (4.7)

Note. Six-week-old male BALB/c mice were inoculated intracerebrally with 10^3 PFU of each variant and clinically observed for 1 week.

[a] No. dead/No. tested.

[b] In days.

the MAb-resistant variants with deletions in the S1 of MHV-4 (Fazakerley *et al.,* 1992) or JHM-DL (Wang *et al.,* 1992) were extremely low neurovirulent for mice compared with the parental virus. This discrepancy was shown not to be due to the difference in mouse strain used in this study. These differences observed among JHMVs maintained in different laboratories might be explained by the differences in the S gene. However, it seems to be more conceivable that the genetic differences in viral genes outside the S gene such as the HE gene (Yokomori *et al.,* 1993) or the 3′ noncoding region of the genome (Lavi *et al.,* 1990) or even unidentified regions in the genome of these viruses account for such different neurovirulence for mice.

In the present study, we used MAbs recognizing different epitopes on the S1 protein. We have shown that the MAbs in group B are reactive almost specifically to the S protein of all JHMV variants, while those in group C recognize only a large S protein of JHMV (Kubo *et al.,* 1993). MAbs in the former group were revealed to recognize the epitopes composed of N-terminal 330 aa of the S1, while localizations of epitopes recognized by the MAbs in the latter group were not precisely determined, except for a part of an epitope located in the C-terminal 1/4 of the S1 protein (Kubo *et al.,* 1994). The MAbs in group B selected the variant viruses with a point mutation in the S1 gene, while the variants with a deletion of about 150 aa were selected by the MAbs in group C. It has been reported that the variant viruses with the deleted S protein emerged during the passage of the virus with larger S protein in cultured cells (Gallagher *et al.,* 1990; Parker *et al.,* 1989). We have purified cl-2 by plaque cloning to remove the contaminating variants with deleted S protein and used such virus material as a parental virus for isolating the MAb-resistant variants, but that material could already contain the virus with the deleted S protein. Such minor contaminants could be selected by MAbs 56 and 85.

Recently, it has been reported that the JHMV variants with point mutations in the S2 subunit were selected by the S1-specific MAb (Grosse and Siddell, 1994). This suggested the possibility that our MAb-resistant variants selected by the S1-specific MAbs contain the mutations in the S2 and such mutations may influence the neurovirulence for rats. We have analyzed the S2 nucleotide sequence of MM85 with altered neurovirulence and found no amino acid changes in the S2 compared with that of parental cl-2. This result excludes the possibility that the alteration of neurovirulence of MM85 is due to amino acid mutations in the S2 subunit.

High neurovirulence of JHMV variants without deletion in the S protein could be accounted for by the high replication in the rat brains. However, it is not known at present why such viruses have a growth advantage in the rat brain as shown in the present study. It may be possible that the larger S protein can bind to a cellular receptor expressed specifically in rat brain, in which MHV-specific receptors such as those found in mice (Williams *et al.,* 1991) have not yet been detected. In particular, the region of the S1 deletion in the small S protein may play a critical role for such binding. It seems to be important to identify the cell type in the rat brain which permits cl-2 replication but not that of avirulent variants. Cultivation of such cells *in vitro* will help the molecular analysis of the neurovirulence of JHMV.

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