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VIROLOGY 186, 742–749 (1992)

Sequence Analysis of the Spike Protein Gene of Murine Coronavirus Variants: Study of Genetic Sites Affecting Neuropathogenicity

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Received August 19, 1991; accepted November 1, 1991

Mouse hepatitis virus (MHV), a coronavirus, causes encephalitis and demyelination in susceptible rodents. Previous investigations have shown that the MHV spike (S) protein is a critical determinant of viral tropism and pathogenicity in mice and rats. To understand the molecular basis of MHV neuropathogenesis, we studied the spike protein gene sequences of several neutralization-resistant variants of the JHM strain of MHV, which were selected with monoclonal antibodies (MAbs) specific for the S protein. We found that variant 2.2-V-1, which was selected with MAb J.2.2 and primarily caused demyelination, had a single point mutation at nucleotide (NT) 3340, as compared to the parental JHM virus, which predominantly caused encephalitis. This site was in the S2 subunit of the S protein. In contrast, variant 7.2-V-1, which was selected with MAb J.7.2 and primarily caused encephalitis, had two point mutations at NT 1766 and 1950, which were in the S1 subunit. Finally, the double mutant 2.2/7.2-V-2, which was selected with both MAbs J.2.2 and J.7.2, and was attenuated with respect to both virulence and the ability to cause demyelination, had a deletion spanning from NT 1523 to 1624 in the S1 and a point mutation at NT 3340 in the S2. We conclude that at least two regions of the S protein contribute to neuropathogenicity of MHV. We have also isolated a partial revertant of 2.2-V-1, which was partially resistant to MAb J.2.2 but retained the same neuropathogenicity as the variant 2.2-V-1. This revertant retained the mutation at NT 3340, but had a second-site mutation at NT 1994, further confirming that NT 3340 contributed to the pathogenic phenotype of MHV. By comparing these results with MHV variants isolated in other laboratories, which had mutations in other sites on the S gene and yet retained the demyelinating ability, we suggest that the ability of JHM viruses to induce demyelination is determined by the interaction of multiple sites on the S gene, rather than the characteristics of a single, unique site. Our study also revealed the possible presence of microheterogeneity of S gene sequence, particularly in the S1 region, in these viruses. The sequence microheterogeneity may also contribute to the differences in their biological properties.

INTRODUCTION

Mouse hepatitis virus (MHV) is a prototypic member of the Coronaviridae. The virus is enveloped and contains a single-stranded, positive-sensed, and nonsegmented RNA genome of 31 kb in length (Lai and Stohlman, 1978; Pachuk *et al.*, 1989; Lee *et al.*, 1991). The virus particle contains three envelope glycoproteins (S, M, and HE) and an internal nucleocapsid protein (N). The S (spike) protein of 180 kDa forms peplomers on the virion surface and is responsible for the viral attachment to cellular receptors, the elicitation of neutralizing antibodies, and the induction of cell-to-cell fusion (Collins *et al.*, 1982; Sturman *et al.*, 1985). Alterations in the S protein resulted in changes in viral pathogenicity, as demonstrated by neutralization-escape mutants selected with monoclonal antibodies (MAbs) specific for the S protein (Fleming *et al.*, 1986, 1987; Dalziel *et al.*, 1986; Wege *et al.*, 1988). Furthermore, passive immunization with S-specific monoclonal antibodies also altered viral neuropathogenicity (Buchmeier *et al.*, 1984). The S protein is cleaved into two 90-kDa glycoprotein subunits (S1 and S2). Its cleavage is carried out by a cellular protease and is probably required for the virus-induced cell fusion (Storz *et al.*, 1981; Yoshikura and Tajima, 1981; Sturman *et al.*, 1985). The S protein is translated from mRNA 3, which represents the transcript of the 4.2-kb gene (S gene), located at roughly 6–10 kb from the 3′-end of the RNA genome (Lai, 1990). The complete sequence of the S gene has been reported for three strains of MHV, namely, A59 (Luytjes *et al.*, 1987), JHM (Schmidt *et al.*, 1987), and MHV-4 (equivalent to JHM used in our laboratory) (Parker *et al.*, 1989). It appears that the S gene sequences of different MHV strains differ significantly, with various degrees of deletion and substitutions.

Infection of the central nervous system (CNS) of rodents by MHV, particularly the JHM strain, has been

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0042-6822/92 $3.00

used as a model system for studying human demyelinating diseases, such as multiple sclerosis. Parental JHM strain shows tropism for both neurons and glial cells, causing both encephalitis and chronic demyelination (Weiner, 1973). The tropism of MHV is mainly determined by the properties of the S protein, since it is the outermost component of the viral envelope, which interacts with the cellular receptors, and it is also the immunodominant component which interacts with the host immune system (Sturman and Holmes, 1983). The role of the other structural proteins in viral pathogenesis is less well understood; passive immunization with MAbs specific for N and M (membrane) proteins also changed viral pathogenicity (Fleming *et al.*, 1989; Nakanaga *et al.*, 1986).

Previously, we have isolated several neutralization-resistant variants of JHM using MAbs specific for the S protein (Fleming *et al.*, 1983). These variants showed strikingly different capabilities from that of the parental JHM virus in causing demyelination or encephalitis in rodents (Fleming *et al.*, 1986, 1987). While the parental virus caused encephalitis, the variants selected with one neutralizing MAb (J.2.2) had lower virulence and caused demyelination, and the variants selected with two MAbs (J.2.2 and J.7.2) caused neither encephalitis nor demyelination (Fleming *et al.*, 1986, 1987). Based on these studies, we hypothesized that MAb J.2.2 identified a specific encephalitis-determining site and MAb J.7.2 identified a specific demyelination-determining site on the S protein. Alternatively, we considered the possibility that a single linear determinant might not be the necessary and sufficient cause of JHM-induced demyelination, and that demyelination would depend on several regions of S or even on other viral genes (Fleming *et al.*, 1987). Our present sequence analysis indicates that the latter hypothesis is correct; that is, the neuropathogenicity of JHM depends on the characteristics of at least two sites on the S gene. Complex interaction of different regions of S protein likely influences viral pathogenesis by changing the degree of viral attenuation *in vivo.*

MATERIALS AND METHODS

Viruses and cells

The viruses used in this study included several JHM variants which were selected for their resistance to neutralizing MAbs specific for the S protein (Fleming *et al.*, 1983). Their virulence and neuropathogenicity in mice are listed in Table 1. The parental virus JHM-DL was a large-plaque isolate of wild-type JHM strain and caused marked encephalitis with chronic demyelination in survivors (Stohlman *et al.*, 1982). The 2.2-V-1 variant was selected from JHM-DL with MAb J.2.2, and caused marked demyelination with little encephalitis (Fleming *et al.*, 1986). The 2.2/7.2-V-2 variant was selected from 2.2-V-1 with another MAb J.7.2 and was nonpathogenic, causing only minimal neuropathology (Fleming *et al.*, 1987). The 7.2-V-1 variant was selected directly from JHM-DL with J.7.2 and remained encephalitic (Fleming *et al.*, 1986). An astrocytoma cell line (DBT cell) derived from mice (Hirano *et al.*, 1974) was used throughout the experiment. All of these viruses were initially plaque-purified three times before propagation as virus stock. Early passage viruses (before passage 3) were used for preparing large amounts of virus for biochemical studies. The viruses of the same passage level were previously used for pathogenicity studies (Fleming *et al.*, 1986, 1987).

TABLE 1

PROPERTIES OF DIFFERENT JHM ISOLATES

Isolates	MAb resistance	Pathogenicity	LD_{50} (PFU)
JHM-DL	—	E >>> D	4
2.2-V-1	J.2.2	D	10^4
2.2/7.2-V-2	J.2.2 + J.7.2	Nonpathogenic	$>10^5$
7.2-V-1	J.7.2	E	25

Note. MAb, Monoclonal antibody; LD_{50}, 50% lethal dose in C57/B6 mice on day 7 after intracerebral inoculation; PFU, plaque forming units; E, predominantly encephalitis; D, predominantly demyelination. (Fleming *et al.*, 1986, 1987).

Growth and purification of viruses

Variant viruses were grown in the presence of the respective MAb used for their selection. Viruses were mixed with an equal amount of MAb tissue culture supernatants and incubated for 45 to 60 min at 37° before inoculating onto confluent DBT cell monolayer. After virus adsorption for 1 hr at 37°, Dulbecco's minimal essential medium containing 1% fetal calf serum was added. The supernatants from infected cell cultures were harvested when the cytopathic effects reached 90–100%. The harvested viruses were examined for their resistance to the selecting MAbs to ensure that the viruses used for biochemical studies maintained the original phenotype. The JHM-DL virus was grown in the same way except that no antibodies were used.

Viruses were purified according to a procedure modified from Makino *et al.* (1983). Briefly, the supernatants were centrifuged at 8000 rpm for 30 min to clear cell debris and were precipitated with 50% saturated am-

monium sulfate for at least 1.5 hr at 4°, followed by centrifugation at 8000 rpm for 20 min. The pellets were suspended in NTE (0.1 *M* NaCl, 0.01 *M* Tris–HCl, pH 7.4, and 1 m*M* EDTA), and the suspensions were centrifuged at 2000 rpm for 10 min to remove undissolved debris. The supernatants were then subjected to two rounds of centrifugation in discontinuous sucrose gradient consisting of 20, 30, 50, and 60% sucrose in NTE at 26,000 rpm for 3.5 hr at 4° in a Beckman SW28 rotor. Viruses at the 30–50% interface were harvested and diluted with NTE and pelleted by centrifugation at 40,000 rpm for 90 min in a Beckman SW41 rotor. The virus pellets were resuspended in NTE.

Isolation of partial revertants of neutralization-escape variant 2.2-V-1

Variant 2.2-V-1 was passaged in DBT cells for 10 times in the absence of any antibodies. These viruses were plaque-assayed, and plaques were randomly picked and further purified by three rounds of plaque purification. Several plaques were examined for their sensitivity to MAb J.2.2. Large pools of revertants were prepared and purified following the same procedure as described above. These plaque-purified revertants were designated as V10.

cDNA cloning

For viral RNA extraction, the virus suspension in NTE was mixed with an equal volume of proteinase K buffer (2% SDS, 0.025 *M* EDTA, 0.2 *M* Tris–HCl, pH 7.5, and 0.3 *M* NaCl) and proteinase K (200 μg/ml), and incubated for 45 min at 37°. RNA was extracted twice with phenol/chloroform (1:1) and precipitated with ethanol. The pelleted RNA was resuspended in distilled H_2O, and stored at −70°.

For cDNA synthesis, viral RNA was first denatured with 10 m*M* methylmercury at room temperature for 10 min and then incubated at 42° for 45 to 60 min in 50 μl of buffer containing 0.01 *M* $MgCl_2$, 0.1 *M* KCl, 0.05 *M* Tris–HCl, pH 8.3, 0.01 *M* dithiothreitol (DTT), 0.03 *M* β-mercaptoethanol, 0.1 m*M* each of deoxyribonucleoside triphosphates (dNTPs), 60 units of ribonuclease inhibitor RNasin (Promega), 0.1 to 1 μ*M* of appropriate synthetic oligonucleotide primer (first primer), and 15 units of avian myeloblastosis virus reverse transcriptase (Boehringer-Manheim). The synthesized cDNA was amplified by polymerase chain reaction (PCR) following a method modified from Saiki *et al.* (1988). Briefly, 10 μl of reverse transcription mixture was mixed with 90 μl of buffer containing 0.05 *M* KCl, 0.01 *M* Tris–HCl, pH 8.3, 2.5 m*M* $MgCl_2$, 0.01% gelatin, 200 μ*M* of dNTPs, 1–3 units of Taq polymerase (Cetus), and 0.1–1 μ*M* of synthetic oligonucleotide primers (first and second primers). The reaction mixture was overlaid with 60 μl of mineral oil and subjected to 20–40 cycles of amplification, each cycle consisting of 94° for 1 min, 37–42° for 1–2 min, and 72° for 2–3 min. These PCR conditions were adjusted slightly for different reactions, depending on the size of target sequence to be amplified and the pair of primers used.

The PCR product was resolved on a 1% agarose gel in 1× TBE buffer (0.089 *M* Tris–HCl, pH 8.3, 0.089 *M* boric acid, and 2 m*M* EDTA) and stained with ethidium bromide. The specific product band was cut out, and the DNA was extracted from the gel with phenol and chloroform, precipitated with ethanol, and the pellet suspended in distilled H_2O. The PCR product was treated with 10 units of polynucleotide kinase (Boehringer-Manheim) in buffer containing 0.07 *M* Tris–HCl, pH 8.0, 0.01 *M* $MgCl_2$, 5 m*M* DTT, 0.1 m*M* spermidine, and 1 m*M* ATP and incubated at 42° for 45 min. After phenol/chloroform extraction and ethanol precipitation, the PCR product was blunt-ended with 1 unit of T4 DNA polymerase (Boehringer-Manheim) at 37° for 30 min in buffer containing 0.05 *M* Tris–HCl, pH 7.5, 7 m*M* $MgCl_2$, 1 m*M* DTT, 0.25 m*M* of dNTPs, followed by a booster reaction with an additional 0.5 unit of T4 DNA polymerase and 0.05 m*M* of dNTPs at 37° for another 30 min. After phenol/chloroform extraction, the PCR product was ligated using 1 unit of T4 DNA ligase (Boehringer-Manheim) into the SmaI site of plasmid pTZ18U (U.S. Biochemicals). The ligation buffer contained 0.05 *M* Tris–HCl, pH 7.5, 0.01 *M* $MgCl_2$, 2 m*M* DTT, and 1 m*M* ATP. The ligation mixture was used to transform competent cell DH5α (BRL) following the manufacturer's direction.

DNA sequencing

DNA sequencing was performed using double-stranded DNA of plasmid pTZ18U. Dideoxy chain termination sequencing was performed following the procedure outlined in the Sequenase version 2.0 DNA sequencing kit from U.S. Biochemical. The reaction product was analyzed in 6% polyacrylamide gel containing 7 *M* urea.

RESULTS

Strategy for cDNA cloning and sequencing

The whole S gene, approximately 4.2 kb in length, was cloned into 2 to 3 overlapping fragments after amplification by PCR using appropriate synthetic primers. Since we were dealing with several viral isolates with presumably only minor differences in S gene, cross-contamination was a major concern. To avoid this problem, whenever possible, different pairs of primers

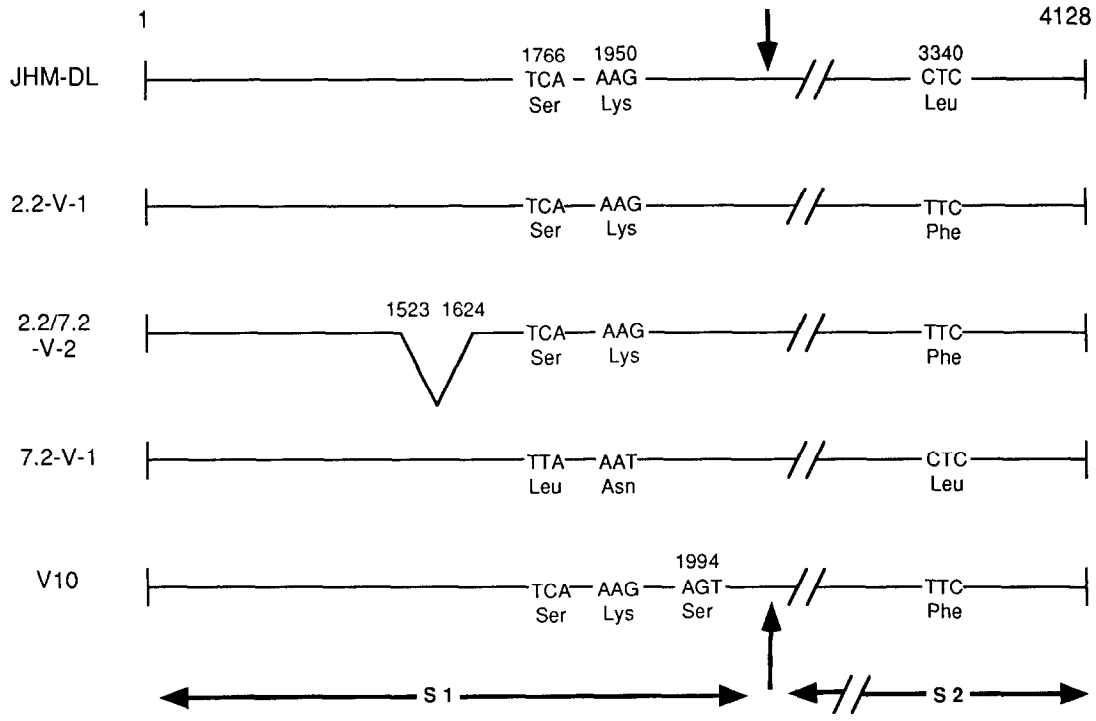

FIG. 1. Schematic summary of the S gene sequence divergence observed among different JHM-DL variants. Only consistent changes are indicated. Numbers on the upper left and right corners define the beginning and the end of S gene nucleotide position. Other numbers indicate the nucleotide positions of point mutations or deletion. Vertical arrows indicate the cleavage site between amino acids 769 (Arg) and 770 (Ser), i.e., between nucleotide 2307 and 2308.

were used in the cloning of different viral RNAs so that distinguishable gene fragments were obtained for each viral isolate. In cases where alternative primers were not available, new aliquots of reagents were used for each isolate. Another concern was that the enzymes used, namely, reverse transcriptase, Taq polymerase, and Sequenase may generate errors. To avoid this problem, clones from different cDNA synthesis events, different PCR events, and multiple clones were sequenced. For each isolate, the full-length sequence was initially obtained from two different DNA clones. Differences in sequence identified by this initial sequencing were further studied by sequencing more clones. Only consistent changes were considered as bona fide mutations. Some of the sequence variations were confirmed by direct RNA sequencing using purified virion RNA (Banner *et al.*, 1990).

The complete S gene sequences of various virus isolates

The complete sequences of S gene of the parental JHM-DL and their neutralization-escape variants are summarized schematically in Fig. 1. The S gene of JHM-DL is nearly identical to that of MHV-4 (Parker *et al.*, 1989) with only 6 nucleotide differences (3 amino acid differences) (Table 2). It has a coding capacity of 1376 amino acids. Identical to those in other MHVs (Schmidt *et al.*, 1987; Luytjes *et al.*, 1987), the predicted proteolytic cleavage site is between amino acid 769 (Arg) and 770 (Ser). The S gene of 2.2-V-1, which was derived from JHM-DL by selection with MAb J.2.2, appeared to consist of two populations. All of the clones contained a nucleotide difference between JHM-DL and 2.2-V-1, which was located at nucleotide (NT) 3340, changing the codon from CTC to TTC (Leu to Phe). Besides this mutation, a minority of clones contained four additional mutations at the N-terminal

TABLE 2

S GENE SEQUENCE DIVERGENCE BETWEEN MHV-4 AND JHM-DL

Nucleotide positions	MHV-4		JHM-DL	
	Codon	(amino acid)	Codon	(amino acid)
763–765	CTG	(Leu)	GCT	(Ala)
893	AGT	(Ser)	ACT	(Thr)
2312	GTT	(Val)	GGT	(Gly)
3990	GCC	(Ala)	GCT	(Ala)

Note. MHV-4 sequence was from Parker *et al.* (1989).

TABLE 3

SEQUENCE VARIATIONS IN JHM-DL AND 2.2-V-1 S GENES

Viruses	Nucleotide position of mutation		Coded amino acid	Frequency
2.2-V-1	731	TTT → TCT	Phe → Ser	2/6
	901	CCG → TCG	Pro → Ser	2/6
	950	GGA → GAA	Gly → Glu	2/6
	1439	CCG → CTG	Pro → Leu	1/6
JHM-DL	65	ATC → ACC	Ile → Thr	2/6
	843	GCG → GCT	Ala → Ala	1/5
	1259	ATC → ACC	Ile → Thr	1/5
	1789	GAT → AAT	His → Asn	1/3
	3282	CAG → CAT	Gln → His	2/15

Note. Sequence variations in 2.2-V-1 were found consistently in two cDNA clones, which were derived from different reverse transcription events and polymerase chain reactions. In contrast, sequence variations in JHM-DL were found randomly in different clones. Frequency indicates the number of clones showing the alternative nucleotide out of the total number of clones that were sequenced.

end of the S gene (Table 3). These were located at NT 731, 901, 950, and 1439, changing the codon from TTT to TCT (Phe to Ser), from CCG to TCG (Pro to Ser), from GGA to GAA (Gly to Glu), and from CCG to CTG (Pro to Leu), respectively. These mutations were not likely to be due to enzyme errors, because these mutations were observed on two clones derived from different reverse transcription and PCR events. Sequencing of multiple JHM-DL-derived clones also showed heterogeneity at several different nucleotide positions (Table 3), which were different from the sequence variations observed in 2.2-V-1. Since the only consistent difference between JHM-DL and 2.2-V-1 was NT 3340, this mutation was most likely responsible for the resistance of 2.2-V-1 to MAb J.2.2. However, the contribution of sequence microheterogeneity (Table 3) to the phenotype of viruses could not be ruled out. It should be noted that most of the minor sequence variations were in the N-terminal half, which was the more divergent part of the S gene (de Groot *et al.,* 1987).

The variant 2.2/7.2-V-2 was derived from 2.2-V-1 by selection with MAb J.7.2 (Fleming *et al.,* 1987). Sequence analysis showed that 2.2/7.2-V-2 had a 102-nucleotide deletion at the N-terminal half between NT 1523 and 1624, corresponding to a 34-amino acid deletion. Accompanying the deletion, the two amino acids arginine and tyrosine at the junction of deletion were converted into histidine. As expected, NT 3340 in this variant remained as TTC, similar to that in 2.2-V-1. Also, similar to 2.2-V-1, a small number of clones contained several additional point mutations at the upstream and downstream regions of the deletion (data not shown). These sequence variations were different from those observed in the variant 2.2-V-1. Since the 102-nucleotide deletion was the only consistent change observed in all clones of 2.2/7.2-V-2, it was most likely responsible for the phenotype differences.

To confirm that the deletion in the N-terminal half of S was responsible for the viral resistance to MAb J.7.2, we sequenced the S gene of 7.2-V-1, which was derived directly from JHM-DL by selection with MAb J.7.2 (Fleming *et al.,* 1986). To our surprise, 7.2-V-1 did not have a deletion. Instead, there were two point mutations consistently detected downstream of the 2.2/7.2-V-2 deletion site, one at NT 1766, changing the codon from TCA to TTA (Ser to Leu) and the other one at NT 1950, changing the codon from AAG to AAT (Lys to Asn). All of these changes were localized around the hypervariable region (de Groot *et al.,* 1987; Parker *et al.,* 1989; Banner *et al.,* 1990) of the S protein. These results suggested that the mutations, including deletions and point mutations, in this region were most likely responsible for the viral resistance to MAb J.7.2. NT 3340 of 7.2-V-1 remained as CTC, similar to that in JHM-DL, consistent with the fact that 7.2-V-1 was directly derived from JHM-DL.

Sequence analysis of a partial revertant of 2.2-V-1

To determine whether the mutation at NT 3340 or the microheterogeneity in S1 was responsible for the viral resistance to MAb J.2.2 and for the pathogenicity of the virus, we attempted to isolate revertants of 2.2-V-1. Variant 2.2-V-1 was passaged in DBT cells in the absence of any MAb, and viruses were randomly selected from plaques. Since the parental JHM-DL grew better than the neutralization-resistant variants (unpublished observations), we reasoned that the revertants would become a predominant population during serial viral passages in the absence of selection pressure. Indeed, most of the plaque-purified viruses isolated this way were partially sensitive to MAb J.2.2 (data not shown). Their titers were reduced by MAb J.2.2 by approximately 2.7 $\log_{10}$. However, none of the viruses examined ($N = 7$) completely reverted to the level seen in parental JHM-DL, whose titer was reduced by MAb J.2.2 by approximately 4 $\log_{10}$ (Fleming *et al.,* 1986, 1987). Sequence analysis of multiple clones from all seven partial revertants showed that all of them have retained the sequence of 2.2-V-1 at NT 3340, which was the only consistent difference observed between JHM-DL and 2.2-V-1. One of these revertants was chosen for complete sequencing; this virus had a consistent change at a second site at NT 1994, changing the codon from AAT (Asn) to AGT (Ser). Since none of the revertants completely restored

the phenotype to that of the parental JHM-DL, the most logical explanation was that different regions of the S protein may cooperate to contribute to the resistance of the virus to neutralization by MAb J.2.2. The finding that NT 3340 mutation was retained in all of the partial revertants suggested that this site had a major contribution to the resistance to MAb J.2.2. Preliminary viral pathogenicity study by intracerebral inoculation in mice revealed that V10 had an LD_{50} of approximately 10^3 PFU, similar to that of 2.2-V-1, but in contrast to JHM-DL, which had an LD_{50} of 4 PFU (Table 1) (Fleming *et al.,* 1986). Similar to 2.2-V-1, the partially revertant virus primarily caused demyelination with little encephalitis. These results suggested that the second site mutation in the S1 region did not alter the pathogenicity of the virus and that the mutation at NT 3340 was most responsible for the neuropathogenicity of the JHM viruses.

DISCUSSION

Our sequence analysis of MAb-escape mutants of JHM indicated that a mutant selected with MAb J.2.2 had a point mutation in the S2 region of the spike protein. By contrast, when MAb J.7.2 was used to select mutants, point mutations or a deletion in S1 within NT 1500–1950 were observed. Previous attempts at mapping the binding sites of these MAbs have failed (Weismiller *et al.,* 1990). Our study suggested that MAb J.7.2 would bind to S1 while MAb J.2.2 would bind to S2. These findings are consistent with a recent study measuring the direct binding of the MHV-specific MAb, which also indicated that J.2.2 bound to S2 (P. Talbot, personal communication). The binding site of J.2.2 is close to other S2 neutralizing epitopes identified in other laboratories (Luytjes *et al.,* 1989; Routledge *et al.,* 1991). Interestingly, the mutation at NT 3340, which changed the coded amino acid from leu to phe affected one of the three amino acids which are critical for the fusion function of the S protein (Gallagher *et al.,* 1991). However, the variant 2.2-V-1 induced fusion when grown on DBT cells (data not shown); this was expected since the loss of the fusion activity required the simultaneous mutations of all the three amino acids (Gallagher *et al.,* 1991). Nevertheless, these findings show that this amino acid plays an important role in both fusion activity and neuropathogenicity of MHV.

The region of S1 with deletion or point mutations in 2.2/7.2-V-2 or 7.2-V-1 mutants corresponds to a previously identified hypervariable site on S1 (Parker *et al.,* 1989; Banner *et al.,* 1990). This site underwent frequent deletions or point mutations upon viral passages in tissue culture or animals (Dalziel *et al.,* 1986; Banner *et al.,* 1990; Gallagher *et al.,* 1990). Although the precise sites of deletion or point mutations were different among these variants, they were in the same general area, i.e., near the C-terminus of S1. Previously, Buchmeier's laboratory also has reported different types of mutation among the neutralization-escape MHV-4 variants, which were derived by use of a monoclonal antibody different from the one used in our study (Parker *et al.,* 1989; Gallagher *et al.,* 1990). All of these variants have either deletion or point mutations in this area of S1. These results together suggested that this region represented an immunodominant and highly variable site, although the binding site of MAb J.7.2 has not been directly determined.

Taken together with previous studies, our analysis indicates that at least two sites on S play critical roles in MHV neuropathogenesis: (1) the hypervariable S1 region within NT 1500–1950 and (2) a second region on S2 around NT 3340. Preservation of the parental (wild-type) sequences at both of these regions appears to be required for the ability of virus to cause fatal encephalitis, since mutations at either site reduced neurovirulence.

The mutation in S2 (2.2-V-1) converted the parental JHM virus, which was highly encephalitic, into a highly demyelinating virus, and an additional mutation (deletion) in S1 (2.2/7.2-V-2) led to an essentially non-demyelinating virus (Fleming *et al.,* 1987) (Table 1). This result was consistent with the interpretation that the site on S1 was tightly associated with demyelination, while the site on S2 was responsible for encephalitis. However, this interpretation is unlikely, since other neutralization-resistant mutants with deletions in the same domain of S1 still caused demyelination (Dalziel *et al.,* 1986; Gallagher *et al.,* 1990). Thus, we hypothesize that the virtual inability of 2.2/7.2-V-2 to cause demyelination or encephalitis (Fleming *et al.,* 1987) was due to viral attenuation that occurred when both the S1 and S2 determinants were lost or mutated. Such an interpretation is consistent with the finding that the mutation in S2 of the variant 2.2-V-1 and the mutation in S1 of other mutants (Dalziel *et al.,* 1986) all led to a similar demyelinating phenotype. This is also consistent with our current understanding of the biological activities of the S protein.

The S protein forms peplomers which project from the surface of the MHV virion, and is thus in an ideal position to influence pathogenesis by altering viral attachment to cellular receptors, by interaction with the immune system, by fusion of cellular membranes (Collins *et al.,* 1982; Sturman *et al.,* 1985), and perhaps by other mechanisms, such as influencing viral stability, uncoating, or spread. After intracerebral inoculation, parental JHM-DL rapidly gains entry to glia and neurons throughout the CNS and thus is able to cause

fatal panencephalitis (Weiner, 1973; Knobler *et al.*, 1981). By contrast, MAb-selected JHM mutants (2.2-V-1 and 2.2/7.2-V-2) are less able to spread to neurons, and virulence is thus drastically reduced (Fleming *et al.*, 1986). However, these viruses are able to infect glial cells of the white matter of the brain and spinal cord. Presumably the requirements of viral entry or replication in glial cells are less stringent than those for neuronal infection. This differential entry may reflect the presence of different receptors utilized by wild-type virus versus selected variants. In addition, the susceptibility of different neural cells to JHM is known to be affected by the developmental stages of different cell species and host factors (Pasick and Dales, 1991). In the case of infection by JHM mutants, the immune response clears virus from the white matter by 12 days postinoculation but has the deleterious consequence of inducing myelin loss (Wang *et al.*, 1990). Thus, the apparent increase in ability to cause demyelination shown by these and similar variants (Dalziel *et al.*, 1986; Wege *et al.*, 1988) is probably due to the unmasking of potentialities of the parental virus which are usually hidden by its virulence.

The ability of mutations at different sites to affect the same biological properties of the S protein was further demonstrated by the sequence analysis of a partial revertant of 2.2-V-1. The second site mutation which partially restored the MAb sensitivity of this virus was localized on S1, in contrast to the original mutation on S2. This study suggested that two sterically separated sites (one in the globular portion, and the other in the heptad repeat region of the stalk portion according to the computer modeling [de Groot *et al.*, 1987]) on the two different S subunits could interact with each other. This effect has also been illustrated by the antibody sensitivity of 2.2/7.2-V-2 and 2.2-V-1. The former was roughly one $\log_{10}$ more resistant than the latter to MAb J.2.2 (Fleming *et al.*, 1987), suggesting that the second mutation further altered the conformation at the first site (in S2). Interestingly, the partial revertant of 2.2-V-1 retained the same pathogenic properties of the parent variant 2.2-V-1. Furthermore, 7.2-V-1 variant retained the ability to cause encephalitis (Table 1). These results suggest that the antibody binding and pathogenic properties of the S protein can be dissociated.

Our study also suggested the possible presence of sequence microheterogeneity in the MHVs (Table 3 and data not shown). All of these viruses were initially plaque-purified but had been amplified by limited passages to obtain sufficient virus titer before molecular cloning and sequencing. Some of these sequence variations were likely the results of PCR and cloning errors. However, several nucleotide variations were consistently noted in repeated PCR products. Furthermore, the sequence microheterogeneity was located mostly in the S1 hypervariable region (Banner *et al.*, 1990; de Groot *et al.*, 1987), suggesting that the observed sequence variations represented variations in the RNA. Since the plaque-purified viruses need to be propagated for either biochemical or pathogenicity studies, this sequence microheterogeneity is likely an inherent property of any virus population. The possible contribution of the sequence microheterogeneity to viral neuropathogenicity is not clear at the present time.

In conclusion, our studies suggest that at least two regions of the S gene play critical roles in JHM neuropathogenesis. These regions are near NT 1500–1950 (S1 segment) and NT 3340 (S2 segment). Antigenic studies indicate that these sites may be closely linked on the S protein and may influence each other by spatial proximity or steric effects (Fleming *et al.*, 1986). Viruses with the parental JHM sequences may infect all cell types and regions of the CNS, resulting in fatal panencephalitis. When either site is altered, viral infection is limited to glial cells of the white matter, and subsequent immune attack (Wang *et al.*, 1990) or virus-induced cytolysis will lead to demyelination. When both sites are affected, the virus shows very limited distribution and presumably is contained by the immune system at an early stage of pathogenesis. It is probable that other S domains may also contribute to the viral pathogenicity. In addition, although the S gene is considered to be the most important in influencing pathogenicity, other viral genes such as hemagglutinin-esterase (HE) (La Monica *et al.*, 1991, Yokomori *et al.*, 1991) and those localized to the 3′ portion of the genome (Lavi *et al.*, 1990) may also, to some extent, contribute to viral pathogenicity.

ACKNOWLEDGMENTS

We thank Drs. Y-C. Chao and C-Z. Lee for technical advice. We also thank Daphne Shimoda for the manuscript preparation. This work was supported by U.S. Public Health Services Grants NS1816 from the National Institutes of Health and RG 2283-A-2 of the National Multiple Sclerosis Society. F.I.W. is a recipient of postdoctoral training fellowship from NIH. M.M.C.L. is an Investigator of Howard Hughes Medical Institute.

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