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VIROLOGY 239, 1–10 (1997)
ARTICLE NO. VY978877

Altered Pathogenesis of a Mutant of the Murine Coronavirus MHV-A59 Is Associated with a Q159L Amino Acid Substitution in the Spike Protein

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Received July 7, 1997; returned to author for revision September 11, 1997; accepted October 1, 1997

C12, an attenuated, fusion delayed, very weakly hepatotropic mutant of mouse hepatitis virus strain A59 (MHV-A59) has been further characterized. We have previously shown that C12 has two amino acid substitutions relative to wild type virus in the spike protein, Q159L (within a region of S1 shown to bind to viral receptor in an *in vitro* assay) and H716D (in the proteolytic cleavage recognition site). We have sequenced the rest of the 31-kb genome of C12 and compared it to wild type virus. Only three additional amino acids substitutions were found, all encoded within the replicase gene. Analysis of C12 *in vivo* in C57Bl/6 mice has shown that despite the fact that this virus replicates in the brain to titers at least as high as wild type and causes acute encephalitis similar to wild type, this virus causes a minimal level of demyelination and only at very high levels of virus inoculation. Thus acute encephalitis is not sufficient for the induction of demyelination by MHV-A59. Analysis of mutants isolated at earlier times from the same persistently infected glial cell culture as C12, as well as mutants isolated from a second independent culture of persistently infected glial cells, suggests that both the weakly demyelinating and the weakly hepatotropic phenotypes of C12 are associated with the Q159L amino acid substitution.

INTRODUCTION

The murine coronavirus, mouse hepatitis virus strain A59 (MHV-A59), produces both hepatitis and neurological disease in susceptible mice. Neurological disease includes both acute meningoencephalitis and chronic demyelinating disease (Lavi *et al.,* 1984b). *In vitro,* MHV-A59 causes a lytic infection in mouse fibroblast cells, but a persistent productive, nonlytic infection of primary cultured glial cells (Lavi *et al.,* 1987). The mechanism of demyelination is not well understood but it is thought to involve elements of direct viral infection as well as an immune mediated component (Houtman and Fleming, 1996a).

We have been using a group of MHV-A59 mutants with altered pathogenic properties to map the determinants of central nervous system pathogenesis and to understand what features of infection of mice lead to demyelination. We have previously characterized this group of attenuated, fusion-delayed mutants, which were isolated from persistently infected glial cells. These mutants had greatly reduced ability to replicate in the liver and cause hepatitis (Hingley *et al.,* 1994).

In order to begin to identify the mutations responsible for the altered pathogenic properties of the mutant viruses, we compared the sequence of the spike (S) genes of wild type and mutant viruses isolated from persistently infected glial cells cultures. We focused on the S protein because this protein mediates both binding to the viral receptor (Holmes and Compton, 1995) and cell to cell fusion. This, in combination with the fact that viruses with mutations in the S gene have altered pathogenic properties (Fleming *et al.,* 1986; Gallagher *et al.,* 1990) suggest that S is a major determinant of viral tropism. The fusion-delayed phenotype was shown to result from the substitution of an aspartic acid residue for a histidine (H716D) in the basic sequence that serves as a recognition signal for proteolytic cleavage of the S protein (Gombold *et al.,* 1993). This amino acid substitution prevents cleavage of the 180-kDa precursor S protein into the two approximately 90-kDa subunits, S1 and S2 (Luytjes *et al.,* 1987; Gombold *et al.,* 1993). Aside from the cleavage recognition site mutation (H716D), all the mutant viruses examined contained one, and only one, additional amino acid substitution in S, Q159L, located within the amino terminal portion of the S1 subunit. Analysis of fusion competent revertant viruses, however, showed that attenuation and loss of hepatotropism were not linked to the fusion phenotype and did not map to the H716D amino acid substitution. An association between the Q159L mutation and loss of hepatotropism was demonstrated by the analysis of other mutants isolated from persistently infected cultures (Hingley *et al.,* 1994). This supports the

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0042-6822/97 $25.00

hypothesis that this mutation may play a role in tissue tropism. Interestingly, Q159L lies within the amino terminal 330 amino acids of S1, a region of the spike protein demonstrated to bind to the viral receptor in an *in vitro* assay (Kubo *et al.,* 1994; Suzuki and Taguchi 1996), suggesting the possibility that this amino acid may influence the interaction of the spike protein with the viral receptor.

Earlier studies (see Table 1) also demonstrated that viruses with identical S gene sequences had different virulence and hepatotropism phenotypes (Hingley *et al.,* 1994). Thus, to determine which additional genes may influence pathogenesis, we have sequenced the entire genome of the C12 mutant of MHV-A59, one of the viruses isolated from persistently infected glial cells (Gombold *et al.,* 1993; Hingley *et al.,* 1994). In addition to the two mutations found in the S gene, there were only five other mutations; of these, three resulted in amino acid substitutions, all within the viral replicase gene.

We describe here the further characterization of the C12 mutant. We have shown that despite the fact that C12 replicates in the brain and causes encephalitis similar to that of wild type virus, at a dose of 5000 PFUs (2 wild type LD_{50}), at which wild type virus induces extensive demyelination in all infected animals, C12 does not induce detectable demyelination. Analysis of other mutants isolated at earlier time points from persistently infected glial cell cultures (Hingley *et al.,* 1994) as well as mutants isolated from an independent culture of persistently infected glial cells, suggests that the Q159L mutation, and not the three replicase mutations, is associated with loss of the ability to demyelinate efficiently as well as the loss of the ability to induce hepatotropism.

METHODS

Virus

MHV-A59 was obtained originally from Dr. Lawrence Sturman (Albany, NY). The mutants were plaque purified from the supernatant of either the "C" culture of persistently infected glial cells at 1 week (C3), 6 weeks (C5), 12 weeks (C8), and 16 weeks (C12) postinfection or from the independent "B" culture of persistently infected glial cells at 18 weeks postinfection (B11, B12) and characterized as previously described (Gombold *et al.,* 1993; Hingley *et al.,* 1994, 1995).

Demyelination

Four- to 6-week-old MHV-free C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were inoculated intracerebrally with 10-fold serial dilutions of wild type or mutant MHV-A59. At 30 days postinfection, mice surviving acute infection were perfused with phosphate-buffered saline, followed by 10% buffered formalin. Brains and spinal cords were removed and tissue was embedded in paraffin and sectioned for staining with luxol fast blue to detect plaques indicative of demyelination. For each of five mice at each dose of virus, demyelination was quantitated by examining one spinal cord section (four quadrants) from each of five levels of spinal cord; thus there were 100 quadrants examined for each dose of virus. The experiments were designed so that there would be five animals surviving acute infection even at high doses of virus relative to the LD_{50}; however, in the case of the highest dose of C3 and all doses of B12, there were less than five surviving animals (Figs. 2 and 3). Demyelination was also verified in the brain by staining with luxol fast blue.

Genome sequencing

For sequencing of the viral genome, reverse transcriptase/polymerase chain reaction amplification (RT/PCR) was carried out, using as templates, cytoplasmic RNA extracted from L2 cell monolayers infected with wild type MHV-A59, C12, C3, C5, C8, B11, or B12. Complementary DNA was synthesized using random oligomers as primers. MHV-A59-specific primers were designed to amplify fragments of about 600 base pairs. Double-stranded PCR products were gel purified and analyzed by automated sequencing using the *Taq* dye terminator procedure according to the manufacturer's protocol (Taq DyeDeoxy Terminator Cycle Sequencing kit, Applied Biosystems) and the same primers used for amplification. Each fragment was sequenced in both directions. The entire C12 genome was sequenced; fragments of each of the other viruses were sequenced as described under Results.

RESULTS

Sequence comparison of the genomes of the C12 mutant and MHV-A59

We have shown previously that the spike (S) protein encoded by the C12 mutant has two amino acid substitutions as compared with the wild type S protein. Earlier studies demonstrated that viruses with the identical S protein sequence had different virulence and hepatotropism phenotypes (see Table 1). For example the mutant B11 appeared to be blocked in its spread from the central nervous system to the liver, while the mutant C12 was inhibited in its ability to replicate in hepatocytes even when inoculated directly into the liver (Hingley *et al.,* 1994). The B12 mutant, while nonhepatotropic like C12, retained its virulence (Hingley *et al.,* 1994). Thus, we concluded that these pathogenic properties must also involve genes outside of S. This, in combination with the idea that it is difficult to assign phenotypes to specific sequences in S without knowing if there are any other mutations outside of S, led us to sequence the rest of

TABLE 1

Pathogenic Phenotypes of Mutants Containing Q159L and H716D Encoded in the S Gene

			Hepatitis[c]	
Virus	Virulence[a]	Encephalitis[b]	IH	IC
WT-A59	+	+	+	+
C12	−	+	−	−
B11	−	+	+	−
B12	+	+	−	−

[a] Virulence is defined by lethal dose $(LD)_{50}$ or the amount of virus needed to kill half the weanling C57Bl/6 mice after intracerebral inoculation. A minus sign indicates the LD_{50} is at least 2 $\log_{10}$ greater than wild type. These data were taken from Hingley *et al.* (1994).

[b] Encephalitis is a measurement of both replication in the CNS brain and detection of viral antigen and pathology in brain sections. The data for WT-A59, C12, and B11 are from Hingley *et al.* (1994).

[c] Hepatitis is defined as replication and detection of viral antigen and pathology in liver sections after intrahepatic (IH) or intracerebral (IC) inoculation of mice. A minus sign indicates that virus titer was at least 2 $\log_{10}$ less than wild type. The data for WT-A59, C12, and B11 are from Hingley *et al.* (1994).

the C12 genome to identify other possible mutations that may play a role in these different pathogenicities.

We have sequenced the entire 31-kb genome of C12. Sequencing was carried out by RT/PCR of viral RNA, present in infected cells, as described under Materials and Methods. This generates a consensus sequence because it is derived from the RNA genome and not cloned cDNAs; we compared the C12 sequence with the published sequence for wild type A59 (from our lab as well as from other labs) (Budzilowicz *et al.,* 1985; Budzilowicz and Weiss, 1987; Zoltick *et al.,* 1990; Bonilla *et al.,* 1994; Weiss *et al.,* 1993; Bredenbeek *et al.,* 1990; Luytjes *et al.,* 1987, 1988; Armstrong *et al.,* 1983, 1984). Whenever we found a difference between C12 and the published MHV-A59 wild type sequence, we made a new preparation of cDNA from intracellular C12 RNA, amplified the same double stranded DNA fragment, and verified the C12 sequence. We then determined if our wild type sequence was the same as the published one. The C12 genome had surprisingly few mutations compared to the wild type genome after 16 weeks of culture in glial cells. Figure 1 shows the comparison of the wild type and C12 genomes.

With the exception of the two previously discussed amino acid substitutions in S, H716D and Q159L (Fig. 1), there are no amino acid substitutions in the deduced sequences of any of the structural proteins; this includes spike (S) (Luytjes *et al.,* 1987; Hingley *et al.,* 1994), matrix (M) (Armstrong *et al.,* 1984), nucleocapsid (N) (Armstrong *et al.,* 1983), and small membrane (E) (Budzilowicz and Weiss, 1987) proteins as well as the open reading frames (ORFs) for the I protein, encoded within, but out of frame with, the N protein (Fischer *et al.,* 1997) and the hemagglutinin esterase (HE) protein, which is encoded, but not expressed in MHV-A59 (Luytjes *et al.,* 1988). The sequence of HE of C12 is identical to the published sequence for wild type MHV-A59 (Luytjes *et al.,* 1988) except for a one-nucleotide deletion which causes a frame shift near the end of the ORF, resulting in the addition of eight amino acids to the end of the ORF. This frame shift mutation is also present in our wild type A59. Thus, our wild MHV-A59 has an additional mutation in the HE ORF; this is consistent with the notion that this protein is not essential in the MHV-A59 life cycle *in vitro* or *in vivo* (Luytjes *et al.,* 1988).

The deduced sequences of the nonstructural proteins encoded in ORFs 2a, 4, and 5a are also identical for C12 and wild type virus. However, in the 21-kb sequence of ORF1a and ORF1b of the replicase gene of the C12 genome (Fig. 1), there are three amino acid substitutions compared to wild type virus. There are two substitutions in ORF1a (P1699S and M2196K) and one in ORF1b, R1330S. (The numbers are amino acid numbers starting from the first amino acid in each ORF.) Of the two substitutions in ORF 1a, P1699S is in the predicted papain-like proteinase-2 (PLP-2) domain; the substitution in ORF 1b, R1330S, is in the predicted helicase domain (Lee *et al.,* 1991; Bredenbeek *et al.,* 1990; Bonilla *et al.,* 1994). There is only one silent mutation in the genome of C12; nucleotide 2471 of ORF 1a is U rather than C, which is in the wild type genome.

We have also compared the noncoding regions of C12 and wild type MHV-A59. We have found no differences between C12 and wild type in the 5′ or 3′ noncoding regions. With the exception of the conserved intergenic region preceding the M gene, there were also no differences between C12 and wild type MHV-A59 intergenic sequences. There is one nucleotide change in the intergenic region preceding the M gene. In C12 the sequence is AAUCUAAAC instead of AAUCCAAAC present in wild type MHV-A59 (Budzilowicz *et al.,* 1985).

We examined the early mutants from the C culture for the presence of the three replicase mutations as well as the intergenic nucleotide difference. These include the C3, C5, and C8 mutants isolated at various times after infection (1, 6, and 12 weeks, respectively). Neither the replicase mutations (Table 2) nor the intergenic region mutation were present in any of the early mutants. This suggested that these mutations arose in the culture later than either of the S mutations.

In order to determine if these replicase substitutions were a general characteristic of the attenuated, tropism mutants that arise in persistently infected glial cells, we examined the genomes of two mutants, B11 and B12, isolated from the independent ("B") culture of persistently infected glial cells (Gombold *et al.,* 1993). None of the three replicase mutations were found in either of these

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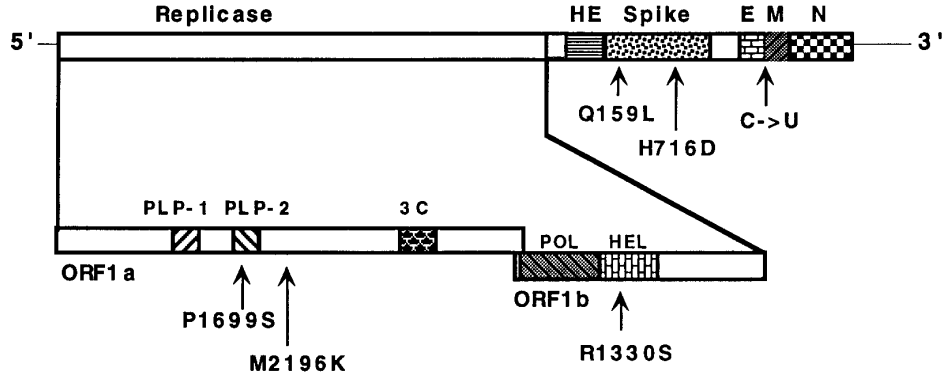

B

Gene	Functional domain	Amino Acid/Nucleotide Substitution
Replicase (ORF1a)	Papain-like proteinase-2	P1699S
Replicase (ORF1a)	Unknown	M2196K
Replicase (ORF1b)	Helicase	R1330S
Spike (S1)	Receptor binding	Q159L
Spike (S1)	Cleavage site	H716D
Intergenic genes 5b/M	Transcription Promoter (?)	AAUCCAAAC to AAUCUAAAC

FIG. 1. Sequence comparison of the C12 and wild type MHV-A59 genomes. The entire C12 genome was sequenced and the sequence compared to that of wild type MHV-A59 (as described in the text). (A) Schematic diagram of the viral genome and the location of the five amino acid substitutions and the one nucleotide change in an intergenic region of C12. The structural proteins are Spike, HE (hemagglutinin esterase), M (matrix), N (nucleocapsid), E (small membrane), and I (internal ORF). The nonstructural replicase proteins are encoded in ORF 1a and ORF 1b. The domains of the replicase PLP-1 (papain-like proteinase-1) and PLP-2 (papain-like proteinase-2), 3C (poliovirus 3C like proteinase), Pol (polymerase), and Hel (helicase) were all predicted by Lee *et al.* (1991). The nonstructural, nonessential protein products of ORFs 2a, 4, and 5a are not shown. The one silent mutation (nucleotide 2471 is U rather than C, present in the wild type genome) is not shown. (B) Genes and predicted functional domains which contain these amino acid substitutions. The amino acids are numbered from the beginning of each open reading frame (ORF) (Bredenbeek *et al.,* 1990; Bonilla *et al.,* 1994; Luytjes *et al.,* 1987). The cleavage site of S was mapped by Luytjes *et al.* (1987) and the mapping of the receptor binding domain is from Kubo *et al.* (1994). The intergenic regions are thought to be involved in transcriptional regulation (van der Most and Spaan, 1995).

genomes. We then sequenced a larger region around the mutation in the predicted PLP-2 domain (1100 nucleotides) and the mutation in the predicted helicase domain (1800 nucleotides) to determine if there were other mutations within either of these predicted domains in B11 or B12. B11 did have another amino acid substitution within the PLP-2 domain, V1798M.

We also found several mutations, both silent and coding, in the comparison of wild type MHV-A59 that we have used to derive the mutants (obtained from Dr. L. Sturman) as compared to the published sequences (Bredenbeek *et al.,* 1990; Bonilla *et al.,* 1994; Luytjes *et al.,* 1987; Luytjes *et al.,* 1988; Hingley *et al.,* 1994; Armstrong *et al.,* 1983, 1984; Budzilowicz *et al.,* 1985; Budzilowicz and Weiss, 1987; Weiss *et al.,* 1993; Zoltick *et al.,* 1990), some of which came from the sequencing of another MHV-A59 (obtained from Dr. J. Leibowitz) in our lab (Bonilla *et al.,* 1994; Budzilowicz *et al.,* 1985; Budzilowicz and Weiss, 1987; Weiss *et al.,* 1993; Zoltick *et al.,* 1990, unpublished sequences of ORF 1b). Mutations encoding amino acid substitutions included two in ORF 1a, two in ORF 1b, one in S, one in N, and one each in ORFs 4 and 5a. Additional silent mutations included three in ORF 1a, two in ORF 1b, and two in N. There were also two nucleotide changes and two insertions in the 3′ noncoding region of the genome.

Demyelination of C12

We have shown previously that the C12 mutant of MHV-A59 has a fusion delayed phenotype *in vitro* (Gombold *et al.,* 1993). Furthermore, C12 replicates to titers equal to wild type virus in the CNS and causes a similar level of encephalitis. However, it is very weakly hepato-

TABLE 2

Pathogenic Properties and Sequences of Mutants Isolated at Various Times from Persistently Infected Glial Cell Cultures

	Amino acid substitutions							
	Spike gene[a]		Replicase gene[b]			Phenotype		
Virus	159	716	1699	2196	1330	Hepatitis[c]	Demyelination[d]	Fusion[e]
WT	Q	H	P	M	R	+	+	+
C3	Q	H	P	M	R	+	+	+
C5	L	H	P	M	R	−	−	+
C8	L	D	P	M	R	−	−	−
C12	L	D	S	K	S	−	−	−
B11	L	D	P	M	R	−	−	−
B12	L	D	P	M	R	−	−	−

[a] The amino acids within the spike protein are numbered beginning with the first residue in the open reading frame (ORF) encoding S.

[b] The amino acids in the replicase are numbered from the first residue of ORF 1a (P1699 and M2196) or the first residue of ORF1b (R1330).

[c] Hepatitis was measured after intracerebral inoculation as described in Table 1.

[d] Demyelination was measured as described under Materials and Methods. A minus sign means that demyelination was minimal as shown in Figs. 2 and 3.

[e] Fusion was defined as previously described Gombold *et al.,* (1993). A negative sign signifies a delayed fusion phenotype in which fusion is negative when measured in L2 cells at 10 h postinfection but eventually does occur by about 24 h postinfection.

tropic (Hingley *et al.,* 1994). In an earlier study (Gombold *et al.,* 1995), we observed that C12 appeared to demyelinate strikingly less well than wild type virus. To assess the difference in demyelination more quantitatively, we measured the amount of demyelination (as described under Materials and Methods) induced by wild type and C12 viruses as a function of dose of virus inoculated. Figure 2 shows these results. For wild type MHV-A59, the amount of demyelination was approximately linear with the $\log_{10}$ of the amount of virus inoculated. With a dose of 5000 PFU (approximately 2 LD_{50}s) of wild type virus, all the animals infected had demyelination (5/5) and demyelination was present in approximately 63% of the spinal cord quadrants examined. We have analyzed demyelination by wild type MHV-A59 in four experiments. The range of demyelination was from 59 to 69% of the quadrants for a dose of 3700 to 5000 PFU; thus the amount of demyelination is quite reproducible. At the same dose C12 caused no detectable demyelination. Even at 100-fold higher dose of inoculation, demyelination was observed in 3/5 of C12 infected animals and in only 13% of the quadrants (Fig. 2). Thus, despite the fact that C12 replicates to high titer in the brain and causes encephalitis to a similar extent as wild type virus (Hingley *et al.,* 1994), it is a poor inducer of demyelination. Similar results were obtained with the B11 and B12 mutants, isolated from an independent culture of persistently infected glial cells (Fig. 2).

Association of demyelination with Q159L

An association between the Q159L substitution in S and the loss of hepatotropism was demonstrated by the analysis of the mutants isolated earlier from persistently infected cultures (Hingley *et al.,* 1994). We have used the same mutants to ask whether demyelination is also associated with this mutation or perhaps with the replicase mutations that arise later during persistence. Thus, the C3, C5, and C8 mutants were analyzed for the ability to induce demyelination. These results are shown in Fig. 3 and summarized in Table 2. The C3 mutant was able to induce demyelination with the same efficiency as wild type virus, while the C5 and C8 viruses exhibit a very weakly demyelinating phenotype, similar to C12. This suggests that the demyelination phenotype correlated with the appearance of the Q159L mutation and before the H716D and the replicase amino acid substitutions in the evolution of the "C" culture.

DISCUSSION

We have previously shown that the group of fusion delayed, nonhepatotropic MHV-A59 mutants isolated from two independently infected glial cells had the same two, and only two, amino acid substitutions relative to wild type in the gene encoding the spike glycoprotein S. The cleavage recognition site amino acid substitution (H716D) clearly correlates with the delayed fusion phenotype, but not with loss of hepatotropism (Hingley *et al.,* 1994; Gombold *et al.,* 1993). The other amino acid substitution, Q159L, appeared to correlate with the hepatotropism phenotype (Hingley *et al.,* 1994, 1995; see Table 2). We concluded this because the Q159L mutation first appeared in virus plaque purified from two independent persistently infected glial cell cultures at 6 weeks postinfection, at the same time that the altered hepatotropism

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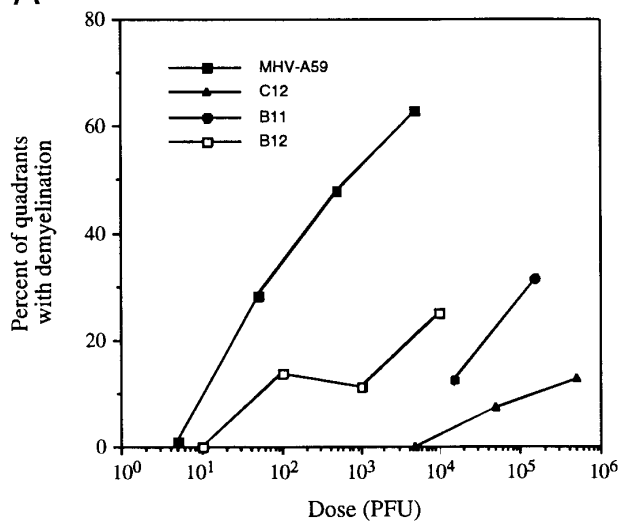

B

Virus	Dose (PFU)	Mice with demyelination/ Total mice	Percent of quadrants with demyelination
MHV-A59	$5x10^0$	1/5	1
	$5x10^1$	3/5	28
	$5x10^2$	5/5	48
	$5x10^3$	5/5	63
C12	$5x10^3$	0/5	0
	$5x10^4$	4/5	8
	$5x10^5$	3/5	13
B11	$1.5x10^4$	2/5	13
	$1.5x10^5$	5/5	32
B12	10^1	0/3	0
	10^2	3/4	14
	10^3	2/3	11
	10^4	4/4	25

FIG. 2. Quantitation of demyelination induced by wild type MHV-A59 and the weakly demyelinating C12, B11 and B12 mutants. C57Bl/6 mice were inoculated intracerebrally with various doses of wild type and mutant viruses. Animals surviving the acute infection were sacrificed at 30 days postinfection, and spinal cords were analyzed for demyelination by staining with luxol fast blue all as described in the text. (A) Amount of demyelination, expressed as the percentage of spinal cord quadrants containing demyelination, at each dose of virus inoculum. (B) Data used in the graph in A. Demyelination values were rounded to the nearest whole number. (In this experiment all animals that were given the highest dose of C12 and those at all doses of B12 survived acute infection).

phenotype became evident. The H716D mutation did not appear until 12 weeks postinfection, along with the delayed fusion phenotype (Hingley *et al.,* 1994; Table 2).

It was not possible to map unambiguously the hepatotropic phenotype to Q159L when the sequence of the rest of the genome was not known. Furthermore, the observation that C12, B11, and B12 have somewhat different pathogenic properties while possessing the same sequence of S (Table 1; Hingley *et al.,* 1994) suggested that mutations outside of S must influence the pathogenesis of this virus. Since there is not yet available an infectious cDNA clone for MHV, we were not able to introduce individual mutations into the viral genome. Therefore, we used an alternative method to map mutations that alter pathogenic properties; we sequenced the entire 31-kb genome of C12.

Surprisingly, there were only five amino acid substitutions in the entire 31-kb genome of C12 after 16 weeks of persistent culture in glial cells. This suggests that the fidelity of the MHV polymerase is higher than commonly

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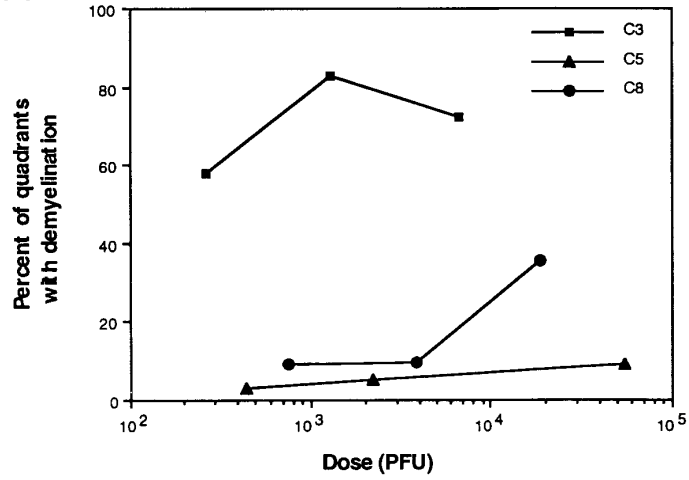

B

Virus	Dose (PFU)	Mice with demyelination/ Total mice	Percent of quadrants with demyelination
C3	$2.7x10^2$	5/5	58
	$1.3x10^3$	5/5	83
	$6.6x10^3$	2/2	73
C5	$4.4x10^2$	1/5	3
	$2.2x10^3$	1/5	5
	$5.5x10^4$	5/5	9
C8	$7.6x10^2$	2/5	9
	$3.8x10^3$	3/5	10
	$1.9x10^4$	5/5	36

FIG. 3. Quantitation of demyelination induced by the "early" C3, C5, and C8 mutants. C57Bl/6 mice were inoculated intracerebrally with various doses of mutant viruses. Animals surviving the acute infection were sacrificed at 30 days postinfection, and spinal cords were analyzed for demyelination by staining with luxol fast blue all as described in the text. (A) Amount of demyelination, expressed as the percentage of spinal cord quadrants containing demyelination, at each dose of virus inoculum. (B) Data used in the graph in A. Demyelination values were rounded to the nearest whole number. (In this experiment all animals except three that were given the highest dose of C3 survived acute infection).

assumed (Lai, 1990; Adami *et al.,* 1995) and/or that, during persistence in glial cell cultures, there is a strong selective pressure against mutation within the genome. Consistent with this observation was our finding that, in the comparison of our MHV-A59 lab strain with the published sequences, there were just 6 amino acid substitutions and 11 silent mutations. We do not think these sequences differences between the wild type MHV-A59 parent of C12 and the published sequences are significant in terms of pathogenesis. The MHV-A59 used as our wild type in these experiments (obtained from Dr. L. Sturman) exhibits the same pathogenic properties as the MHV-A59 (obtained from Dr. J. Leibowitz) that we used for our previous pathogenesis studies (Lavi *et al.,* 1984b) and for sequencing of genes 1, 2a, 4, and 5 (Bonilla *et al.,* 1994; Budzilowicz and Weiss, 1987; Weiss *et al.,* 1993; Zoltick *et al.,* 1990).

The only amino acid substitutions in the C12 genome other than the two encoded in the S gene were three amino acid substitutions in the replicase gene. There are two amino acid substitutions encoded in ORF1a (P1699S and M2196K) and one in ORF 1b, R1330S. The P1699S substitution is in the predicted PLP-2 domain of ORF 1a and the R1330S substitution is in the predicted helicase domain of ORF 1b (Bredenbeek *et al.,* 1990; Lee *et al.,* 1991) (see Fig. 1). It is difficult to predict whether the mutation in PLP-2 is likely to be significant. The B11 mutant also has a mutation, albeit a different one from

C12, V1798M in PLP-2. Numerous attempts in our lab to detect an activity for PLP-2 had been unsuccessful, suggesting that the PLP-2 domain does not encode an active proteinase; if this were the case, then mutations in this region of the replicase gene would not be expected to have a phenotype. However, recently, we have found that a recombinant protein encoded in the predicted PLP-2 domain does indeed have a proteinase activity (H. Teng and S. R. Weiss, unpublished results). Thus, if PLP-2 is a necessary activity for replication, these mutations may be significant. The C12 mutation in ORF 1b is in the predicted helicase domain. The recent demonstration of an ATPase activity for a recombinant protein encoded in the predicted helicase domain of ORF 1b of the human coronavirus 229E (Heusipp *et al.,* 1997) supports the prediction that this is indeed a helicase domain. It is likely that such a polypeptide might interact with host cell proteins and thus influence tropism. The third mutation is in ORF 1a, M2196KL, in a region of the replicase that has not been assigned a function.

The AAUCUAAAC sequence observed preceding the M gene of C12 (different from the wild type AAUCCAAAC) is the sequence found in most of the intergenic regions in the wild type genome (Budzilowicz *et al.,* 1985; Bonilla *et al.,* 1994; Luytjes *et al.,* 1987). Thus it is clear that this sequence can function as an intergenic region. Since the mechanism of generation of coronavirus mRNA is still not clearly understood, the role of coronavirus conserved intergenic regions is also not known. However, it is believed that intergenic regions play a role in the control of subgenomic mRNA transcription, perhaps serving as promoters for subgenomic mRNA synthesis (van der Most and Spaan, 1995). Thus, it is possible that this nucleotide change could effect transcription of the C12 mRNA encoding the M gene. We doubt that this is the case as the levels of M protein in cells infected with C12 is approximately the same as in wild type infected cells (unpublished data). While there is no evidence suggesting that any of the mutations in the C12 genome, either silent or encoding amino acid substitutions, can produce a change in replication or pathogenesis by effecting the secondary structure of the genome RNA, we cannot completely rule out this possibility.

We show here that the appearance of the Q159L mutation within the "C" culture of persistently infected glial cells, in addition to correlating with the loss of hepatotropism, also coincides with the development of the weakly demyelinating phenotype. Further evidence that the Q159L mutation is important in the demyelination phenotype comes from our observations that the B11 and B12 mutants, isolated from another parallel glial cell culture, are also weakly demyelinating (Fig. 2) and contain this mutation in the S gene (Hingley *et al.,* 1994).

Thus it appears that the demyelination and hepatotropism phenotypes of C12 are both associated with Q159L, rather than the replicase mutations. The relationship of Q159L to virulence is, however, not as clear. The observation that the B12 mutant retains its virulence (Hingley *et al.,* 1994, Table 1) demonstrates that Q159L is not sufficient for attenuation and furthermore that the loss of hepatotropism does not necessarily lead to attenuation. The hepatotropic, demyelinating C3 mutant also retains its virulence while the nonhepatotropic, weakly demyelinating C5 and C8 mutants are somewhat attenuated although probably not as attenuated as C12 (data not shown). Thus there may be other mutations in these viruses, absent in C12, that modify the virulence phenotype. The data discussed above and shown in Table 1 suggest there must be mutations outside of S that result in the differences in hepatotropism between B11 and C12, and differences in virulence between B12 and the other mutants. It is possible that the three replicase mutations in C12 modify the pathogenic properties. We cannot be sure of the effects of these mutations until we can analyze recombinant viruses containing only the Q159L mutation; such work is in progress.

The mechanism of MHV induced demyelination is to this date still not well understood. There is mounting evidence that both immune mediated components as well as direct infection of virus are factors in the establishment of demyelination (Houtman and Fleming, 1996a). Our observation that for wild type MHV-A59 the amount of demyelination was proportional to the $\log_{10}$ of the virus inoculum (Fig. 2) argues that the amount of virus in the CNS is important in the development of demyelination. This is consistent with the hypothesis that a direct infection mechanism plays a significant role in demyelination (Houtman and Fleming, 1996a). Alternatively, the larger virus inoculum could result in a larger number of persistently infected cells in the CNS to be damaged by the immune response.

MHV acute infection includes encephalitis and virus replication at high titer in the CNS. It has been shown that while infectious virus cannot be recovered from the CNS after the acute infection, viral RNA persists in the white matter of the CNS probably throughout the lifetime of the mouse (Lavi *et al.,* 1984a; Adami *et al.,* 1995). The group of mutants that we are working with all cause acute encephalitis, but at least in the case of the three that we have examined carefully (C12, B11, B12), there are only minimal levels of demyelination and only at very high levels of virus inoculated. Thus encephalitis does not necessarily lead to demyelination. Several other labs have reported mutants of the JHM strain of MHV, in which encephalitis and demyelination are dissociated. For example the attenuated JHM2.2-V-1 mutant (Fleming *et al.,* 1986, 1987) and the MHV-4 mutant 5A13.5 mutant (Dalziel *et al.,* 1986; Fazakerley *et al.,* 1992) cause little encephalitis but do induce significant demyelinating disease. Both of these mutants were selected by resistance to mono-

clonal antibodies directed against epitopes in the S gene; thus, the change in pathogenic phenotype is likely to map to S. The mutants we have selected from persistently infected glial cells (C12, B11, and B12 for example) are, to our knowledge, the first examples of MHV mutants in which encephalitis occurs without subsequent demyelinating disease.

The location of the Q159L amino acid substitution within the predicted receptor binding domain (Kubo *et al.,* 1994) of S suggests that this mutation may play a role in the development of demyelination and hepatitis by affecting the interaction of S and the viral receptor. The inability of C12 to interact with the receptor on one or more liver cell types would be a reasonable explanation for the lack of hepatitis following infection with C12. The difference in the ability of C12 and wild type virus to induce demyelination could also be related to the tropism for a certain cell type. While we have shown that C12 replicates at least to wild type levels in the CNS during acute infection and is found in neurons with a similar localization in the limbic system (Hingley *et al.,* 1994), we are currently studying the spread of the two viruses to various cell types and the persistence in the CNS after the acute disease and viral clearance occurs.

The region defined as a receptor binding domain includes the amino terminal 330 amino acids of S1 (Kubo *et al.,* 1994). Amino acid Q159, and the region surrounding it, is conserved in 9/10 MHV strains (Suzuki and Taguchi, 1996); interestingly the MHV-S strain, a strain exhibiting low pathogenicity, has the same Q159L substitution observed in our mutants. There is, however, no direct evidence that Q159L is involved in receptor interaction; the Q159L amino acid substitution could effect hepatotropism and demyelination via another mechanism, perhaps due to a step in replication after binding and entry.

Another possible explanation for the different pathogenic phenotypes of C12, B11, and B12, as compared with wild type MHV-A59, is that an amino acid substitution in the S protein could result in an altered immune response and this would then result in a change in the ability to induce hepatitis and demyelination. This is a possibility as there is clearly an immune mediated component in MHV induced demyelination (Fleming *et al.,* 1993; Kyuwa *et al.,* 1991; Houtman and Fleming, 1996b). However, we think that this is unlikely because it is difficult to imagine how the Q159L mutation might effect the immune response to MHV as there is no evidence for an epitope recognized by either a $CD4^+$ T cell or a $CD8^+$ T cell in the receptor binding region (the region around the Q159L mutation) of S of the JHM virus (Stanley Perlman, pers. commun.). Thus we think that it is unlikely that the altered demyelination phenotype of C12, B11, and B12 is due to an altered immune response but rather to a change in viral tropism, perhaps through receptor utilization.

We are currently using targeted recombination technology developed by Dr. Paul Masters (Masters *et al.,* 1994; Fischer *et al.,* 1997) to introduce Q159L alone into the genome of wild type MHV-A59. This will allow us to determine unambiguously the effects of this amino acid substitution alone on pathogenesis and interaction of the mutant protein with the MHV receptor.

ACKNOWLEDGMENTS

This work was supported by public health service Grants NS-21954 (S.R.W.) and NS-30606 (S.R.W.) as well as Grants RG-2585A4/1 (S.R.W.) and RG-26151/2 (E.L.) from the National Multiple Sclerosis Society. We thank Drs. Julian Leibowitz and Henry Teng for reading and commenting on the manuscript.

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