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Sequence analysis of the S gene of recombinant MHV-2/A59 coronaviruses reveals three candidate mutations associated with demyelination and hepatitis

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Coronaviruses, mouse hepatitis virus (MHV) strains, exhibit various degrees of neurotropism and hepatotropism following intracerebral (IC) infection of 4-week-old C57Bl/6 mice. Whereas MHV-A59 produces acute meningitis, encephalitis, hepatitis, and chronic demyelination, a closely related strain, MHV-2, produces only acute meningitis and hepatitis. We previously reported that the spike glycoprotein gene of MHV contains determinants of demyelination and hepatitis. To further investigate the site of demyelination and hepatitis determinants within the S gene, we sequenced the S gene of several nondemyelinating recombinant viruses. We found that three encephalitis-positive, demyelination-negative, hepatitis-negative recombinant viruses have an MHV-A59-derived S gene, which contains three identical point mutations (I375M, L652I, and T1087N). One or more of the sites of these mutations in the MHV-A59 genome are likely to contribute to demyelination and hepatitis. *Journal of NeuroVirology* (2001) **7,** 432–436.

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Introduction

Mouse hepatitis Virus (MHV) is a member of the coronavirus family of the *nidovirales* order (Cavanagh, 1997; Lai and Cavanagh, 1997). Nidoviruses produce a variety of hepatic, enteric, and neurologic diseases in animals, and upper respiratory and enteric diseases in humans (Lavi and Weiss, 1989; Lai and Cavanagh, 1997; Lavi *et al*, 1999). Infection of MHV-A59 in 4-week-old C57Bl/6 (B6) mice produces a biphasic disease with acute hepatitis and meningoencephalitis followed by chronic inflammatory demyelinating disease (Lavi *et al*, 1984; Lavi *et al*, 1986). It serves as an excellent laboratory model of virus-induced demyelination, which mimics many of the clinical and pathologic features of multiple sclerosis (MS) (Weiner, 1973; Knobler *et al*, 1981; Wege *et al*, 1982; Lavi *et al*, 1984; Perlman *et al*, 1990; Houtman and Fleming, 1996).

We have recently studied the pathogenesis and complete sequence analysis of the MHV-2 genome, a closely related strain to MHV-A59 (Das Sarma *et al*, 2001). Previous studies suggested that MHV-2 is only weakly neurotropic (Hirano *et al*, 1974; Hirano *et al*, 1981; Wege *et al*, 1981; Yokomori *et al*, 1991; Yamada *et al*, 1997). We found that MHV-2 produces severe hepatitis and meningitis, without encephalitis or demyelination and without persistence of virus in the CNS (Das Sarma *et al*, 2000; Das Sarma *et al*, 2001). Using targeted recombination, we replaced the S gene of MHV-A59 with that of MHV-2, and produced recombinant viruses (Penn98-1 and Penn98-2) that

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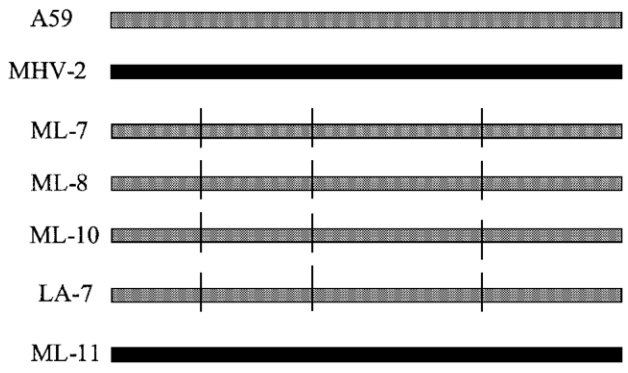

Figure 1 Schematic representation of the S genes of MHV-A59, (light rectangles), MHV-2, (dark rectangles), and the recombinants ML-7, ML-8, ML-10, and ML-11. In the recombinant viruses light rectangles represent MHV-A59-derived sequences and dark rectangles represent MHV-2-derived sequences. The three mutations in ML-7, ML-8, ML-10 and LA-7 are represented by lines.

have a persistence-positive, demyelination-negative phenotype (Das Sarma *et al*, 2000). We concluded that demyelination determinants, independent of viral persistence, map to the S gene of MHV.

To further explore which parts of the S gene are directly responsible for the demyelinating phenotype, we sought to analyze the S gene of viruses that are closely related to MHV-A59, but are unable to produce demyelination. Thus, we used recombinant viruses (ML-7, ML-8, ML-10, and ML-11) that were previously produced by co-infection of cultures with MHV-2 and a temperature-sensitive (ts) mutant of MHV-A59 (LA-7) (Keck *et al*, 1988) (Figure 1). These recombinant viruses were found to be demyelination-negative in preliminary studies.

We first ascertained that the recombinant viruses were able to replicate well in tissue culture. Infection of L2 cells revealed that all recombinant viruses reached titers of approximately 2×10^7 PFU/ml in 24 h. The viruses ML-7, ML-8, and ML-10 produced large plaques and syncytia formation in over 80% of the cultured L2 cells at the peak of lytic infection (similar to MHV-A59). ML-11 produced small plaques and only small syncytia of no more than 3–5 nuclei in less than 5% of the cells during lytic infection (similar to MHV-2).

We then tested the pathogenic properties and histopathology of the recombinant viruses in mice. The virulence of each one of the MHVs in mice was determined by the dose that killed 50% of the mice (LD_{50}) (Reed and Muench, 1938). As previously described, the LD_{50} of MHV-A59 is 4000 PFU, and that of MHV-2 is 200 PFU. The LD_{50} of ML-11 was 4000 PFU and the LD_{50} of ML-7, ML-8, ML-10, and LA-7 was nonlethal (>50,000 PFU). To further assess the kinetics of viral infection in mice, 4-week-old, virus-free, B6 mice were inoculated into the left hemisphere with 1000 PFU (25 μl volume) of each virus. At intervals of 1, 3, 5, 7, 9, 11, 13, and 30 days p.i., 2–4 mice per time point were sacrificed, and perfused with 10 ml PBS, followed by 10 ml 10% buffered formalin. Organs such as the thymus, liver, brain, and spinal cord were removed and fixed in formalin for additional 48 h. Tissues were embedded in paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin (H&E). Brain and spinal cord sections were also stained with Luxol Fast Blue (LFB) for detection of myelin. Each brain was serially sectioned into 5–7 coronal sections. The 5–7 cross-sections were prepared, representing cervical, thoracic, and lumbar regions of the spinal cord. To better demonstrate primary demyelination (loss of myelin sheaths surrounding intact neuronal axons), some mice were perfused with 2% glutaraldehyde; spinal cord sections were embedded in Epon and stained with toluidine blue (Lavi *et al*, 1984).

Table 1 Pathogenesis of recombinant viruses as compared to MHV-A59 and MHV-2

	Meningitis	*Encephalitis*	*Demyelination*	*Hepatitis*
A59	+	+	+	+
MHV-2	+	−	−	+
ML-7	+	+	−	−
ML-8	+	+	−	−
ML-10	+	+	−	−
ML-11	+	+	rare	+

Table 1 summarizes the pathologic findings in mice infected with the different viruses. Following IC injection of 4-week-old B/6 mice, 1000 PFU of MHV-A59 produced moderate acute hepatitis and acute meningo-encephalitis in 14/14 mice, followed by chronic CNS demyelinating disease in 16/16 mice as previously described (Lavi *et al*, 1988; Lavi *et al*, 1990). Infection (IC) of 4-week-old B/6 mice with 1000 PFU of MHV-2 produced severe hepatitis, and meningitis in 14/14 surviving mice without encephalitis as previously described (Das Sarma *et al*, 2001). Because the LD_{50} of MHV-2 is much lower than MHV-A59, we also infected mice with lower doses of MHV-2. Infection of mice with 100 PFU and 200 PFU of MHV-2 produced the same results as 1000 PFU. There was also no demyelination in 0/16 mice infected with 100, 200, and 1000 PFU of MHV-2. Recombinant ML-11 produced acute encephalitis and hepatitis in 7/7 mice, similar to MHV-A59, following IC injection with 1000 PFU. The same dose (1000 PFU) of ML-7, ML-8, and ML-10 produced mild acute encephalitis (in 4/4 mice with each virus), but did not produce hepatitis (in 0/4 mice with each virus). Higher doses of ML-7, ML-8, and ML-10 (up to 2.5×10^5 PFU per mouse) remained nonlethal and produced similar pathologic changes. Viral titers in the brains of mice showed that ML-11 had similar or higher titers than MHV-A59, whereas MHV-2 had lower titers, attributed to the meningitis. ML-10 had low titers (Figure 2). Thymic cortical depletion was observed in MHV-A59, MHV-2, and ML-11 infections. Infection of mice with LA-7 (one of the parental viruses used for the recombination process) did not produce any pathology including no

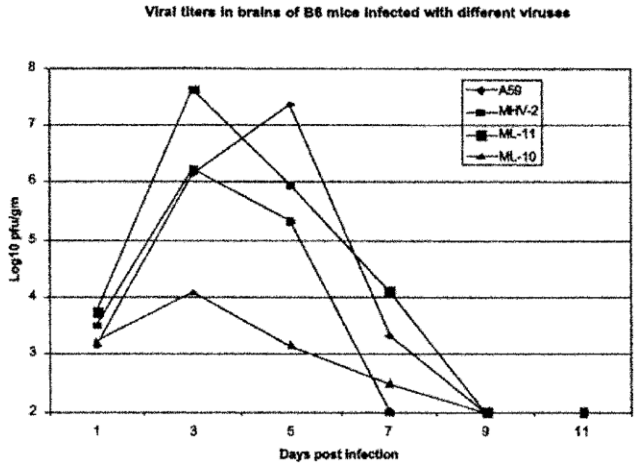

Figure 2 Viral titers in the brains of 4-week-old C57Bl/6 mice during the acute disease following infection with MHV-A59, MHV-2, ML-11, ML10.

demyelination. This phenotype is probably because LA-7 is a temperature-sensitive mutant that does not grow very well in mice. The recombinant viruses produced from LA-7 corrected the ts defect and are no longer temperature-sensitive.

Multiple sections of spinal cord from mice infected with the various recombinant viruses were examined for myelin loss and inflammatory lesions at 30 days p.i. MHV-A59 caused chronic spinal cord demyelination that was detected in 16/16 mice by H&E-, LFB-, or toluidine-blue-stained sections. However, mice infected with MHV-2 (0/14), ML-7 (0/3), ML-8 (0/3), ML-10 (0/3), or ML-11 (0/6) did not exhibit chronic demyelination in any of the spinal cord or brain sections stained with H&E and LFB. Toluidine blue staining of Epon-embedded sections revealed numerous demyelinated axons in MHV-A59-infected mice (Figure 3), but no demyelination was seen in mice infected with MHV-2 or with the recombinant viruses, with the exception of ML-11. In mice infected with ML-11, rare demyelinated axons in multiple spinal cord sections were detected in one of the mice (Figure 3). Demyelination was not observed in mice infected with the attenuated viruses (ML-7, ML-8, and ML-10) at doses as high as 2.5×10^5 PFU.

For sequencing of the viral genomes, we used reverse-transcriptase-PCR (RT-PCR) amplification of cytoplasmic RNA extracted from virus-infected L2 cells (m.o.i. = 1), harvested 16 h p.i. Complementary DNA was synthesized using oligonucleotide primers. Primers were designed to amplify fragments of approximately 600 base pairs (Leparc-Goffart *et al*, 1997; Das Sarma *et al*, 2000). The sequencing data was submitted to GenBank (accession numbers AF208067 and AF207902). Sequencing of the S gene of ML-7, ML-8, and ML-10 revealed 3 amino acid substitutions I375M, L652I, and T1087N as compared to MHV-A59 (Figure 1). Because these viruses were derived from a recombination between a ts mutant of A59 (LA7) and MHV-2 (Keck *et al*, 1988), we sequenced the S gene of LA7. We found that LA-7 contained the exact same mutations as the recombinant viruses ML-7, ML-8, and ML-10, indicating that these mutations were all derived from LA7. The amino acid sequence of the S gene of ML-11 was identical to that of MHV-2 (GenBank accession number AF201929). Thus, demyelination-negative viruses had S genes

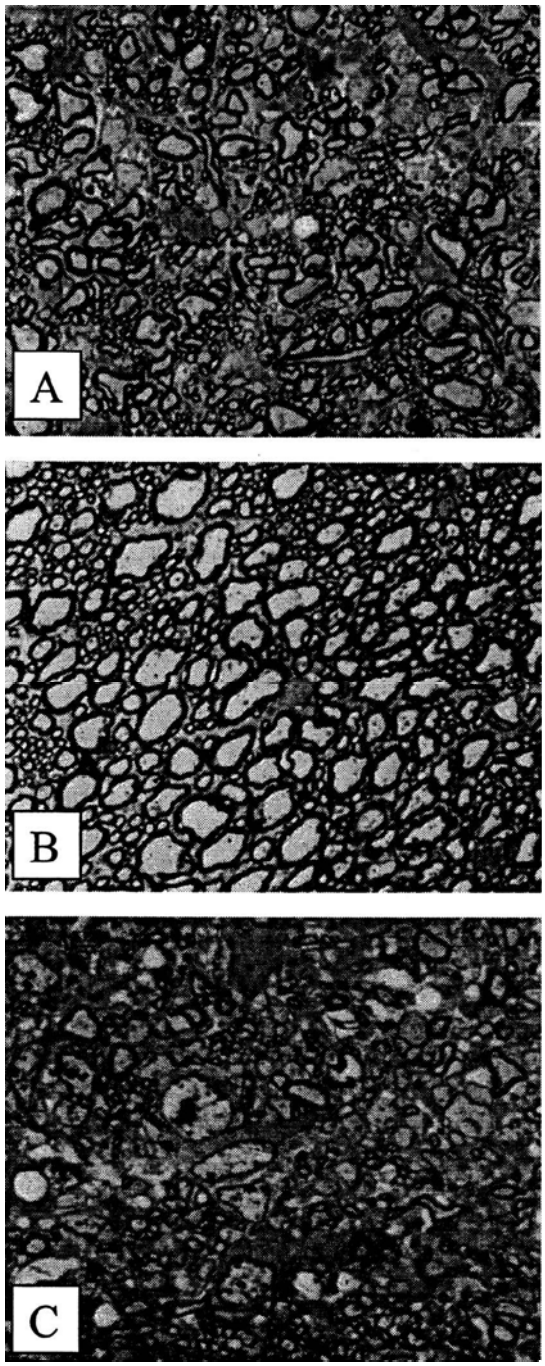

Figure 3 Spinal cord sections of mice infected with 1000 PFU of ML-11 (A), ML-10 (B), and MHV-A59 (C). Sections were embedded in Epon and stained with toluidine blue. Note occasional demyelinated axon in ML-11 infected cord (arrow), normal myelin in ML-10 infected cord, and numerous demyelinated axons in MHV-A59 infected cord.

that belonged to one of two types. These viruses either had an S gene derived from MHV-2, or an S gene derived from A59 but contained the 3 mutations that were acquired from their parental virus LA-7. The hepatitis-negative phenotype also mapped to one or more of the S gene mutations. It has recently been found that determinants of hepatitis also map to the S gene of MHV (Navas *et al*, 2001). Although it is possible that only one of these mutations is relevant to the biologic property of demyelination or hepatitis, two or even all three of these mutations may be necessary to produce these biologic properties. Studies are underway to determine the relative contribution of each one of these mutations to the demyelination-negative and/or hepatitis-negative phenotype. The site of these mutations within the S gene involves various parts of the S gene. The I375M mutation is downstream from the receptor-binding site, the L652I mutation is within the variable region of the S gene and the T1087N mutation is in the region between the two heptad repeats. The specific functions of all of these regions are still unknown. Mutations, in the heptad repeat region at positions 1067 (Q to H), 1094 (Q to H), and 1114 (L to R), have been shown to affect pH-dependent fusion properties (Gallagher *et al*, 1991).

All recombinant viruses produced various degrees of encephalitis but none of them was capable of producing demyelination. This finding suggests that the mutations found in the S gene are not associated with encephalitis, or neurotropism as a general phenomenon, but specifically with demyelination and hepatitis. Because the amount of virus during acute infection was either higher than MHV-A59 (ML-11) or lower than MHV-A59 (ML-10), the level of virus during acute infection is probably not a significant determinant of demyelination. Additional support for this theory comes from studies in which CD28 knockout mice were infected with MHV-A59. The transgenic mutation lowered the level of viral titers during acute infection but did not change the level of demyelination (Das Sarma *et al*, in preparation). It is also clear that whereas demyelination probably depends on various sites in the viral genome, the S gene probably contains the most important determinants of demyelination. Random mutations produced in various parts of the genome are unable to abrogate demyelination (data not shown). The abrogation of the property of demyelination only occurs when specific sites are mutated. So far, only mutations within the S gene have been associated with abrogation of demyelination. Moreover, although acute encephalitis may be a prerequisite for chronic demyelination, the presence of acute encephalitis is not sufficient to induce chronic demyelination. The property of demyelination probably requires additional factor(s) beyond the mere ability of the virus to invade and infect the CNS. These factors may be related to the interaction between the virus and the immune system or may be associated with the ability of the virus to induce certain host responses such as cytokine release or apoptosis. Current studies in our laboratory focus on the factors necessary for encephalitic viruses to be able to produce chronic demyelination.

Previous studies using monoclonal antibodies to modify pathogenic properties, and phenotype-sequence correlation of mutant and variant viruses, linked determinants of pathogenesis to the S, M, and HE proteins (Collins *et al*, 1982; Knobler *et al*, 1982; Buchmeier *et al*, 1984; Dalziel *et al*, 1986; Fleming *et al*, 1989; Lavi *et al*, 1990; Laude *et al*, 1992; Hingley *et al*, 1994; Leparc-Goffart *et al*, 1997). However, the recently adopted targeted recombination for pathogenesis studies of MHV provides more direct and reliable information about molecular determinants of pathogenesis (Lavi *et al*, 1998a; Lavi *et al*, 1998b; Leparc-Goffart *et al*, 1998; Phillips *et al*, 1999; Das Sarma *et al*, 2000).

In conclusion, we describe the sequence analysis of the S gene of several non-demyelinating MHVs. Sequence analysis identifies three S gene mutations that are common to 3 demyelination-negative viruses with an S gene derived from MHV-A59. Therefore, these mutations can be potential candidate sites for direct targeted recombination analysis used to study the mechanism of MHV-induced demyelination. The formation of a virus with an A59 S gene on an MHV-2 background would also be extremely helpful. Site-directed mutagenesis studies in the S gene are underway in our laboratory.

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