

Neuropathogenicity of mouse hepatitis virus JHM isolates differing in hemagglutinin-esterase protein expression

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The hemagglutinin-esterase (HE) protein of mouse hepatitis virus (MHV) is an optional envelope protein present in only some MHV isolates. Its expression is regulated by the copy number of a UCUAA pentanucleotide sequence present in the leader sequence of the viral genomic RNA. The functional significance of this viral protein so far is not clear. In this report, we compared the neuropathogenicity of two MHV isolates, JHM(2) and JHM(3), which express different amounts of HE protein. Intracerebral inoculation of these two viruses into C57BL/6 mice showed that JHM(2), which expresses an abundant amount of HE protein, was more neurovirulent than JHM(3), which expresses very little HE. Histopathology showed that early in infection, JHM(2) infected primarily neurons, while JHM(3) infected mainly glial cells. JHM(3) eventually infected neurons and caused a delayed death relative to JHM(2)-infected mice, suggesting that the progression of JHM(3) infection in the central nervous system was slower than JHM(2). *In vitro* infection of JHM(3) in primary mixed glial cell cultures of astrocyte-enriched cultures yielded higher virus titers than JHM(2), mimicking the preferential growth of JHM(3) in glial cells *in vivo*. These findings suggest that the reduced neuropathogenicity of JHM(3) may correlate with its preferential growth in glial cells. Sequence analysis showed that the S genes of these two viruses are identical, thus ruling out the S gene as the cause of the difference in neuropathogenicity between these two viruses. We conclude that the HE protein contributes to viral neuropathogenicity by influencing either the rate of virus spread, viral cell tropism or both.

Keywords: mouse hepatitis virus; neurovirulence; hemagglutinin-esterase protein; astrocyte culture

Introduction

Mouse hepatitis virus (MHV) is a prototypic member of the Coronaviridae. The neurotropic strain of MHV, JHM virus (MHV-4), has been used as a model for studying both subacute and chronic demyelination (Weiner, 1973; Sorensen *et al*, 1980). It also has the potential to cause acute encephalitis, accompanied by significant loss of neurons. Survivors of acute JHM virus infection often develop subacute or chronic demyelination in the brain, spinal cord, and optic nerves, as a result of oligodendrocyte infection (Lampert *et al*, 1973; Knobler *et al*, 1981;

Stohlman and Winer, 1981).

MHV is an enveloped virus with a positive-sensed RNA genome of 31 kilobases (Pachuk *et al*, 1989; Lee *et al*, 1991). The virion is composed of three major indispensable structural proteins (S, M, and N) and an accessory protein (HE). The S, or spike, protein, which forms major surface projections of viral particles, is responsible for virus-cell interaction, causes fusion of the infected cells and is the binding site for neutralizing antibodies (Collins *et al*, 1982; Sturman *et al*, 1985; Kubo *et al*, 1994). Infection with neutralization-escape mutants, which contain mutations or deletions within the S gene (Parker *et al*, 1989; Gallagher *et al*, 1990; Wang *et al*, 1992), exhibit reduced neurovirulence with minimal encephalitis and increased demyelination (Dalziel *et al*, 1986; Fleming *et al*, 1986). Furthermore, passive transfer into mice with S-pro-

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tein-specific monoclonal antibodies (MAbs) inhibits JHM-induced encephalitis, resulting in a predominantly demyelinating disease (Buchmeier *et al*, 1984; Wege *et al*, 1988). Thus, S protein appears to play a major role in determining the neurotropism of MHV. The M, or membrane, protein interacts with the viral nucleocapsids during virion assembly (Sturman *et al*, 1980). The N, or nucleocapsid, protein interacts with the virion RNA (Stohlman *et al*, 1988) to form nucleocapsid. Passive transfer into mice of non-neutralizing MAbs specific for either the M or N proteins also alters the pathogenicity of MHV (Nakanaga *et al*, 1986; Fleming *et al*, 1989). More recently, a minor envelope protein (gene 5b product) also has been shown to be an indispensable viral protein (Yu *et al*, 1994).

In addition to these proteins, some strains of MHV contain an additional envelope protein, hemagglutinin-esterase (HE) protein, which possesses an esterase activity (Shieh *et al*, 1989; Yokomori *et al*, 1989; Yokomori *et al*, 1991) and a hemadsorption activity (Pfleiderer *et al*, 1991). The esterase activity has also been associated with the HE protein of other coronaviruses, such as bovine coronavirus (BCV) (Vlasak *et al*, 1988a; Vlasak *et al*, 1988b). The HE-specific MAbs of BCV exhibit neutralizing activities (Deregt and Babiuk, 1987). Furthermore, an esterase inhibitor, DFP, has been shown to inhibit BCV infection (Vlasak *et al*, 1988a), suggesting that HE protein is involved in BCV binding to the target cells and is required for BCV infection; however, the inhibitory activity of DFP on BCV infectivity is controversial (Storz *et al*, 1992). In contrast, the HE protein is present in only certain strains of MHV, and HE-specific MAbs of MHV do not neutralize viral infectivity (Yokomori *et al*, 1992); therefore, HE protein of MHV is not required for viral infections. Its biological significance remains unclear.

The MHV proteins are translated from virus-specific mRNAs, which have a 3'-coterminal nested-set structure (Lai *et al*, 1981; Leibowitz *et al*, 1981). In general, only the 5'-terminal unique region of each mRNA is used for translation (Leibowitz *et al*, 1982; Siddell, 1983). The HE protein is translated from an mRNA designated 2-1 (Shieh *et al*, 1989; Yokomori *et al*, 1989), the transcription of which is regulated by the interaction between the leader RNA at the 5'-end of the genomic RNA and the intergenic region upstream of the gene (Shieh *et al*, 1989; Zhang *et al*, 1994). We have obtained two different isolates of JHM virus: the original JHM virus is composed predominantly of a virus designated JHM(3), which contains three copies of a UCUAA pentanucleotide sequence at the 3'-end of the leader RNA (Makino and Lai, 1989). After serial undiluted passages of this virus in tissue culture, a virus population with two UCUAA copies, designated JHM(2), became predominant (Makino and Lai, 1989). Interestingly, JHM(3) virus makes a small amount of mRNA 2-1,

resulting in the synthesis of a small amount of the HE protein (Shieh *et al*, 1989; Yokomori *et al*, 1989); in contrast, JHM(2) virus makes a large amount of mRNA 2-1 and HE protein. Correspondingly, JHM(2) exhibits a higher esterase activity than JHM(3) (Yokomori *et al*, 1989).

Since HE is a viral envelope glycoprotein, it is likely to contribute to virus-cell interactions, even though it is not required for viral infection *in vitro*. Thus, HE protein may play a role in viral pathogenesis. Indeed, passive transfer into mice of HE-specific MAb alters neurotropism of MHV strains that express an HE protein (Yokomori *et al*, 1992).

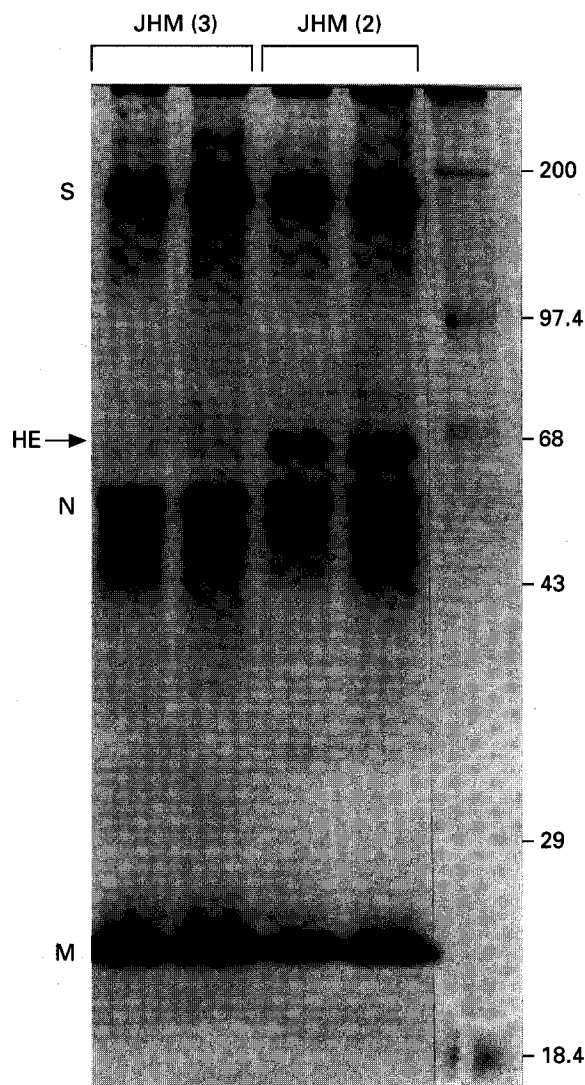


Figure 1 Polyacrylamide gel electrophoresis of viral structural proteins. Two isolates each of JHM(2) and JHM(3) viruses were metabolically labeled with ^{35}S -methionine, and intracellular proteins were precipitated with antibodies against the JHM virus particles (Yokomori *et al*, 1989). The proteins were separated by electrophoresis on 7.5 to 15% gradient polyacrylamide gels. Molecular size markers (in kilodaltons) are indicated.

Therefore, it is predicted that the presence or absence of HE protein in different MHV isolates may alter the biological or pathogenic properties of these viruses. Indeed, a JHM variant isolated from neural cell culture has been reported to express a larger amount of HE protein than the parental virus (Taguchi *et al*, 1986). The current report presents evidence that JHM(2) and JHM(3) viruses have different neuropathogenic properties, and exhibit different growth properties in primary glial cell cultures, suggesting the possible role of the HE protein in the cellular tropism and growth properties of MHV *in vivo*.

Results

Neurovirulence of JHM(2) and JHM(3) viruses in mice

To examine the possible effects on viral neurovirulence of the copy number of UCUAA pentanucleotide in the genomic leader RNA and the accompanying variation of HE gene expression, we plaque-purified four independent isolates of the JHM(2) and JHM(3) viruses. These viruses differed in amount of HE protein expressed, while all other viral structural proteins were indistinguishable: JHM(2) synthesized a large amount of HE protein, whereas JHM(3) synthesized only a trace amount. The protein profiles of two independent isolates of each virus are shown in Figure 1. Groups of mice were injected with 100 PFU of each isolate and the clinical course examined. All mice infected with the JHM(2) isolates showed signs of encephalitis and died on day 7 postinfection (p.i.). In contrast, mice infected with the JHM(3) isolates showed a significant delay in the onset of acute disease. The majority of JHM(3)-infected mice showed clinical signs of encephalitis and died between days 11 and 14 p.i.; however, some of them survived until day 16. All four independent isolates of JHM(2) and JHM(3) gave essentially identical results.

Comparison of virus titers in various organs

To determine the biological basis for the difference in neurovirulence between JHM(2) and JHM(3) viruses, virus replication in brain, spleen and liver of the infected mice were examined on days 1, 3, 5, 7, 10 and 14 p.i. Virus titers in the three organs examined did not differ significantly between JHM(2)- and JHM(3)-infected mice. Figure 2 shows the virus titers in the brain. The virus titers on day 3 and day 5 ranged from 1×10^5 to 10^6 plaque forming units (PFU) per gram of tissue for both JHM(2) and JHM(3). Most significantly, even on day 7, when all JHM(2)-infected mice died, the virus titer in JHM(2)-infected mouse brain was still comparable to that in JHM(3)-infected mice. These data suggested that the difference in neurovirulence of these two viruses is not due to differences in their ability to replicate *in vivo*.

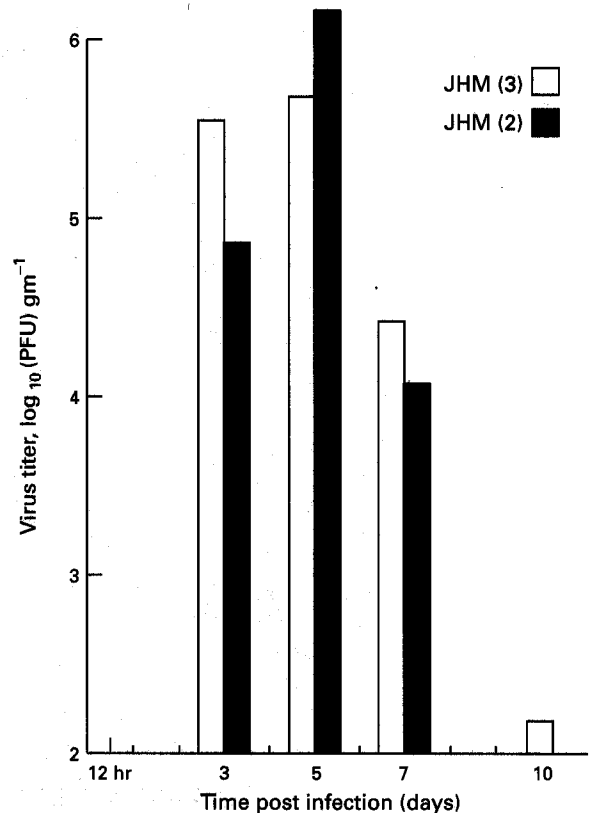


Figure 2 Virus titers in mouse brain during infection. Titters represent the averages from three mice at every time point and are expressed as PFU per gram of tissue.

Histopathology of infected mouse brain

The histopathology and distribution of viral antigens were examined to identify a possible basis for the differences in neurovirulence between these two viruses. Early in infection, the pathological changes in the brains of mice infected with JHM(2) and JHM(3) were indistinguishable and similar to previous descriptions (Yokomori *et al*, 1992). However, on days 5 and 7, the brains of JHM(2)-infected mice showed typical signs of encephalitis, including neuronal necrosis, but showed only minor pathological findings, including demyelination, within the white matter. In contrast, the brains of mice infected with JHM(3) showed more noticeable white matter lesions, including demyelination, associated with abundant inflammatory cellular infiltrates. On day 14 p.i., JHM(3)-infected brain showed evidence of severe encephalitis with significant neuronal necrosis, similar to the pathology observed in JHM(2)-infected mice on day 7 p.i. (data not shown). Thus, JHM(3) infection appeared to cause encephalitis at a slower rate than JHM(2), but in both cases, death was associated with similar pathology, namely, acute encephalomyelitis with demyelination (data not shown; see Yokomori *et al*, 1992).

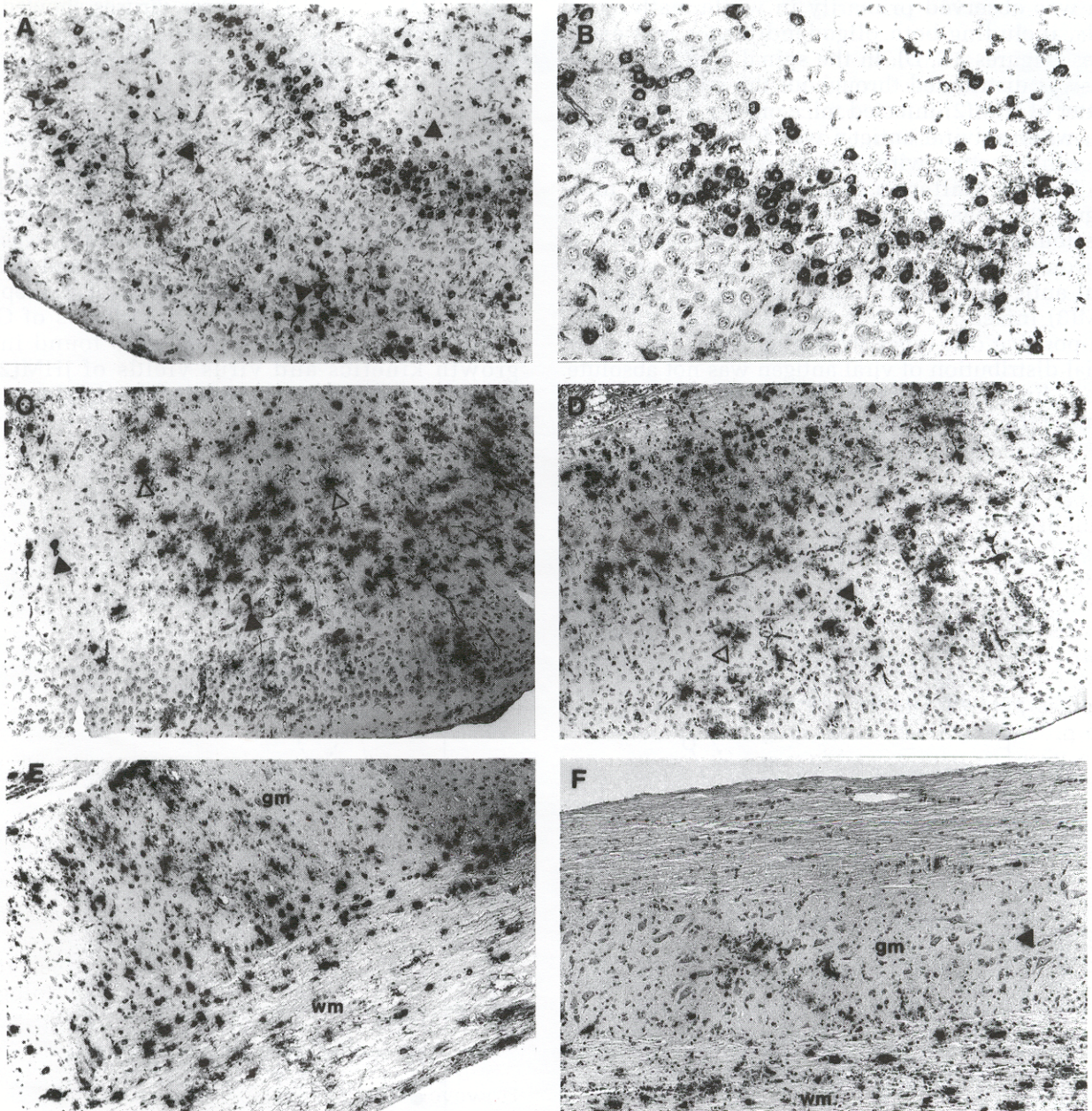


Figure 3 Distribution of viral antigen in JHM(2)- and JHM(3)-infected mouse brain and spinal cord on day 5 p.i. Immunoperoxidase staining was performed using a monoclonal antibody specific for the MHV N protein. (A) Viral antigen in the cortex and hippocampus of JHM(2)-infected mouse brain is found predominantly in neurons (arrowheads). Final magnification = 400 \times . (B) Higher magnification of the same area as in A. Again, the majority of cells staining for viral antigen are neurons. Final magnification = 850 \times . (C) Viral antigen in the brain of JHM(3)-infected mice. Although some neurons show staining (solid arrowheads), the predominantly stained cells are glial (open arrowheads). Final magnification = 310 \times . (D) Same as in C, with final magnification of 350 \times . (E) Viral antigen in the spinal cord of a mouse infected with JHM(2). The majority of stained cells in both white matter (wm) and grey matter (gm) are glial cells. Final magnification = 330 \times . (F) Viral antigen in the spinal cord of a JHM(3)-infected mouse. Most of the stained cells are found in the ventral funiculus of the white matter (wm). Staining in grey matter (gm) largely spares neurons (arrowhead). Final magnification = 360 \times .

To determine whether there were differences in the distribution of viral antigen in the central nervous system (CNS) between JHM(2)- and JHM(3)-infected mice, immunoperoxidase staining of the brain and spinal cord (see Materials and Methods)

was performed using a MAb specific for the N protein. On days 5 and 7 p.i., viral antigen was found predominantly in neurons of the cerebral cortex and hippocampus of JHM(2)-infected mice (Figure 3A, B). In contrast, in JHM(3)-infected mice, viral anti-

gen was observed primarily in white matter glial cells, including both oligodendrocytes and astrocytes (Figures 3C, D). In the spinal cord, viral antigen was distributed throughout the grey and white matter of JHM(2)-infected mice on day 5 p.i. (Figure 3E), whereas viral antigen was found primarily in the white matter, with only occasional focal staining in grey matter in the JHM(3)-infected mice (Figure 3F). It is important to note that viral antigen was found predominantly, but not exclusively, in neurons in mice infected with JHM(2), and that viral antigen was also found in neurons following JHM(3) infection, though the predominantly antigen-positive cell types were glia. Thus, the preferential distribution of viral antigen was not absolute. These findings indicated that, although the virus titers in the CNS were similar in mice infected with

either JHM(2) or JHM(3), these viruses showed different patterns of viral antigen distribution, suggesting differences in either the rate of viral spread or in cellular tropism.

Growth properties of the viruses in cell culture in vitro

The histopathological studies showed that JHM(2) and JHM(3) viruses were distributed differently within the CNS. To determine whether this was due to differences in the ability of JHM(2) and JHM(3) viruses to grow in different cell types, virus replication was examined in *in vitro* cultures of CNS-derived cells. No differences were found in the growth kinetics and virus yields of JHM(2) or JHM(3) viruses in a murine oligodendroglioma cell line (G26.20) (Sundarraj *et al*, 1975) or an astrocy-

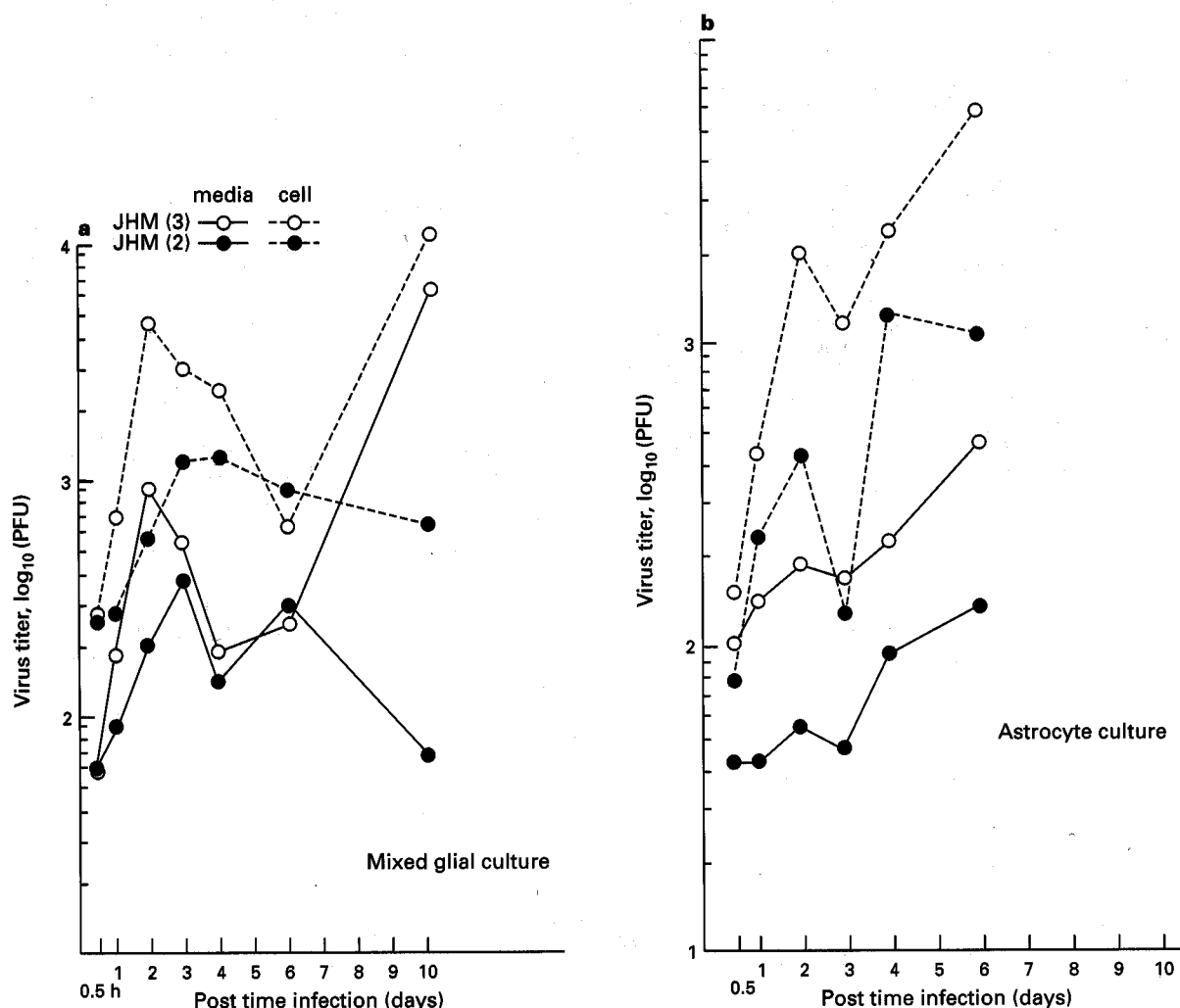


Figure 4 Time course study of virus yields from cell lines and primary glial cell cultures. JHM(2) and JHM(3) were inoculated onto different cell cultures at an m.o.i. of approximately 10. Virus titers in media and cells were determined on the days indicated. Cell-associated virus was released from trypsinized cells by freezing and thawing three times. Virus titer was determined by plaque assay on DBT cells. 'Cell' denotes cell-associated virus; 'media' denotes virus released in the media. Virus titers are expressed as PFU per ml. Symbols are the same for both panels. (A) Primary mixed glial cell culture. (B) Primary astrocyte cell culture.

toma cell line (DBT) (Hirano *et al*, 1974) (data not shown). In contrast, in primary murine mixed glial cell cultures, JHM(3) virus yielded slightly higher titers than JHM(2) in both released virus and cell-associated virus throughout the culture period (10 days) (Figure 4A). Virus titers peaked at 2–3 days post-infection and gradually declined thereafter. But, in some cultures, virus titers recovered on day 5 or 6. The reason for this fluctuation is not clear. Nevertheless, JHM(3) virus titer was always higher than JHM(2) virus.

To determine whether this difference was due to differential growth of the JHM(2) and JHM(3) viruses in oligodendrocytes or astrocytes, virus replication was examined in glial cell cultures enriched for astrocytes (95%). Similar to the mixed glial cell cultures, JHM(3) replicated slightly better than JHM(2) virus in the astrocyte-enriched culture throughout the culture period (6 days) (Figure 4B). Although the differences between the two viruses in both the released virus and cell-associated virus titers were small (approximately $1 \log_{10}$ PFU ml^{-1}), they were reproducible in three different cell preparations with three different pairs of independently isolated JHM(2) and JHM(3) viruses (Figure 4). Similar to the mixed glial cell culture, virus titers peaked on day 2, declined thereafter, but recovered later in the infection in primary astrocyte culture. Nevertheless, similar differences between the titers of JHM(2) and JHM(3) viruses were maintained throughout. This differential replication of JHM(2) and JHM(3) viruses in primary astrocyte culture is consistent with the *in vivo* viral infection data, which showed that JHM(3) antigen was distributed preferentially in the white matter, suggesting the preferential growth of JHM(3) virus in glial cells.

Leader RNA sequence of the viruses isolated from the infected brain

The results described above demonstrated that, on day 14 p.i., JHM(3)-infected mice showed histopathological changes similar to those of JHM(2)-infected mice on day 7 p.i. Thus, it is possible that JHM(3) virus might have evolved *in vivo* into JHM(2) virus during CNS infection. To examine this possibility, virus isolates from the brains of JHM(3)-infected mice obtained on day 14 p.i. were examined for the copy number of UCUSA repeats at the 5'-end of the genomic RNA by a primer extension analysis (Makino *et al*, 1988). Only a single primer-extended product corresponding to the presence of three UCUSA copies was detected (data not shown), indicating that no conversion of JHM(3) virus into JHM(2) occurred in the brain during the 14-day infection period. Thus, the virological and histopathological properties of JHM(3)-infected mice observed at day 14 p.i. do not represent an *in vivo* reversion to the JHM(2) genotype.

Sequence comparison of the S gene of JHM(2) and JHM(3) viruses

The major structural difference between JHM(2) and JHM(3) is the amount of HE protein in the virion (Figure 1). However, the differences in their pathogenicity are similar to those observed between JHM virus and its neutralization-escape mutants, which have mutations in the S gene (Dalziel *et al*, 1986; Fleming *et al*, 1986; Fazakerley *et al*, 1992). To rule out the possible presence of alterations in the S protein, the S genes of both viruses were cloned and sequenced. The data showed that the S gene sequence of the two JHM virus isolates is almost identical to that of the published DL strain of JHM

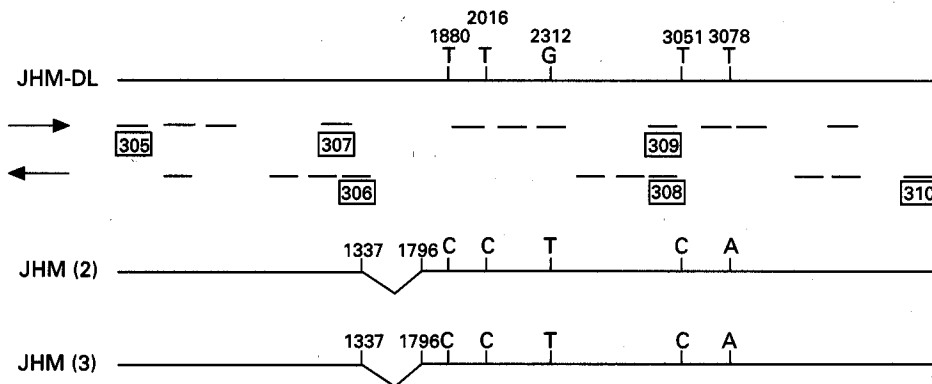


Figure 5 The schematic cloning and sequencing strategy of the S gene from JHM(2) and JHM(3) and the summary of the sequences. The short horizontal lines indicate the positions of the primers used for cDNA cloning and sequencing. The numbered primers were used for cDNA cloning. S gene was cloned into three cDNA fragments using primer combinations of 305–306, 307–308 and 309–310, respectively. Arrows indicate the sequence direction of the primers. The S gene sequence of JHM was from Wang *et al*, (1992). The deletions and nucleotide substitutions in the JHM(2) and JHM(3) S genes are indicated.

(Wang *et al*, 1992), except for a deletion of 459 nucleotides and five single-nucleotide substitutions (Figure 5). Most importantly, the S gene sequences of JHM(2) and JHM(3) are completely identical (Figure 5). Since the amount of the S protein is also similar between the two viruses (Figure 1), these data suggest that the difference in HE protein expression is most likely responsible for the difference in the neuropathogenic properties of these viruses observed in this study.

Discussion

The data shown in this report suggest that differences in the expression of the HE protein between JHM(2) and JHM(3) isolates correlates with their neurovirulence and histopathology of the CNS in mice. Since the major detectable structural difference between these two viruses is the amount of HE protein, it is very likely that the HE protein is responsible for these biological properties. The amounts of other major viral structural proteins in these two viruses are equivalent. Furthermore, the sequence of the S gene, which has previously been shown to be the major determinant of viral pathogenicity (Dalziel *et al*, 1986; Fleming *et al*, 1986; Wege *et al*, 1988; Fazakerley *et al*, 1992), is identical between the two viruses. Although it cannot be rigorously ruled out that alterations in the viral nonstructural proteins may contribute to the observed differences, these data, and the ability of HE MAb to protect mice from acute disease (Yokomori *et al*, 1992) are consistent with a role of the HE protein in MHV pathogenesis. Since the HE protein contains an esterase activity (Vlasak *et al*, 1988a; Yokomori *et al*, 1989), which is similar to the receptor-destroying activity of influenza C virus (Vlasak *et al*, 1988b), it is conceivable that an abundance of HE in JHM(2) virus could facilitate virus spread during infection, similar to the postulated functions of HE or neuraminidase of influenza viruses. Thus, the presence of HE protein on the surface of virion and/or infected cells could contribute either directly or indirectly to viral pathogenesis.

The difference in the neurovirulence of these two viruses was reflected in the differential distribution of viral antigen in glial cells and neurons in infected mice, particularly during the early stage of viral infection. JHM(2) preferentially infects neurons, while JHM(3) preferentially infects glial cells. The pathology and patterns of viral antigen distribution of JHM(3)-infected mice was similar to those previously reported for the parental JHM virus-infected mice (Weiner, 1973; Stohlman and Weiner, 1981). JHM(3) infection eventually led to fulminant encephalitis, although at a slower rate than JHM(2)-infected mice. The difference in neurovirulence does not appear to be due to differences in the overall growth rate of virus, since virus titers in the

brain were not significantly different even on day 7 p.i., when JHM(2)-infected mice died. Also, the death of JHM(3)-infected mice on day 14 p.i. was not due to the conversion of JHM(3) to JHM(2) *in vivo*. The immunohistochemical studies showed that virus spread to the cortex from the initial inoculation area, ie, near the lateral ventricle, faster in JHM(2)-infected mice than in JHM(3)-infected mice. Similarly, the rate of viral spread in the spinal cord was also faster in JHM(2)-infected mice (Figure 3). Therefore, JHM(2) and JHM(3) differ in their ability to disseminate within the CNS. Consistent with this hypothesis is a recent observation that overexpression of the HE protein by a recombinant vaccinia virus leads to extensive cell-cell fusion in a variety of established cell lines (unpublished observation). Our data also indicate that JHM(3) grows somewhat better than JHM(2) in glial cells *in vitro*. Therefore, it is likely that differences in both the rate of viral spread and cell tropism between these two viruses contribute to the difference in their pathogenicity. It remains to be determined how the HE protein can effect such differences.

There were more inflammatory cellular infiltrates in the JHM(3)-infected mice than in the JHM(2)-infected mice on days 5 and 7 p.i. It is not clear whether this difference was a result of different localization of virus replication or possible differences in the induction of the host immune response. All JHM(2)-infected mice died approximately on day 7 p.i., when the host adaptive immune response has not fully developed; in contrast, JHM(3)-infected mice died on day 14 p.i., when immune responses are fully active. Thus, there appears to be no causal link between the immune response and death. However, the immune response may contribute at least to the neuropathogenesis of JHM(3). Increased survival of JHM(3)-infected mice was associated with abundant inflammatory cell infiltrates. This result is consistent with the previous conclusions that cellular immunity plays a role in JHM-induced demyelination in the later stages of infection (Wang *et al*, 1990; Fleming *et al*, 1993).

The S protein is critical in determining viral tissue tropism since it interacts with the viral receptor (Collins *et al*, 1982; Kubo *et al*, 1994); however, it is not directly responsible for the differences in virulence or neuropathogenicity between JHM(2) and JHM(3). Nevertheless, the effect of HE protein on viral pathogenesis may depend on the structure and properties of the S protein in each particular virus. Both JHM(2) and JHM(3) used in this study have a truncated S gene, which may reduce the affinity of the S protein for viral receptor. Reduced receptor affinity may enhance the role of the HE protein in virus-cell and cell-cell interactions since HE protein has an esterase activity (Vlasak *et al*, 1988a; Vlasak *et al*, 1988b; Yokomori *et al*, 1989) and a weak hemagglutinin activity (Pfleiderer *et al*, 1991).

Thus, the biological consequence of HE protein expression *in vivo* may be variable, depending upon the genetic background with respect to structure of the S gene. Furthermore, the potential role of other viral genes in the pathogenesis of JHM(2) and JHM(3) cannot be rigorously ruled out. Minor mutations in other genes of these two closely related viruses may have gone undetected. However, since at least three independently isolated virus pairs of JHM(2) and JHM(3) were compared, it is most likely that HE protein is responsible for the differences between these two viruses. This study thus provided a potential functional role for the HE protein of MHV.

Materials and methods

Viruses and cells

Plaque-purified JHM(2), which contains two UCUEA copies at the 3'-end of the leader sequence of the viral genomic RNA, and JHM(3), which contains three UCUEA copies, were isolated after fourteen undiluted passages of the JHM virus stock (Makino and Lai, 1989). Viruses were propagated on DBT cells, a murine astrocytoma cell line (Hirano *et al*, 1974) as described previously (Makino *et al*, 1984). An oligodendroglioma cell line, G26.20 (Sundarraj *et al*, 1975), was used for the study of viral growth properties.

Animals

Six-week-old male C57BL/6 mice, seronegative for MHV, were obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were inoculated with 100 plaque-forming units (PFU) of JHM(2) or JHM(3) viruses intracerebrally (i.c.) in the left hemispheric to a depth of approximately 3 mm. Three mice each were sacrificed on days 1, 3, 5, 7, 10 and 14 p.i. Brain, spinal cord, spleen and liver were collected for histological examination, and virus titers in the brain, spleen and liver were determined as previously described (Yokomori *et al*, 1992).

Virus titration

Viral titers in each tissue were determined by end-point infectivity on mouse L2 cells as described previously (Stohlman and Weiner, 1981). Briefly, tissue samples were homogenized in ice-cold Dulbecco's phosphate-buffered saline (PBS), clarified by centrifugation at $1500 \times g$ for 5 min, and serially diluted in minimum essential medium (MEM) containing 10% tryptose phosphate broth (TPB) and 2% fetal calf serum (FCS). Two hundred microliters of each dilution were inoculated onto confluent L2 cells in 24-well plates. After 2-h adsorption at 37°C, inoculates were removed and 1 ml of DMEM containing 2% FCS was added. Cytopathic effect (CPE) of cells was examined after 24- and 48-h incubation at 37°C.

Histopathology

Tissues were immersion-fixed for 3 h in Clarke's solution (75% absolute alcohol and 25% glacial acetic acid) and embedded in paraffin. Sections of brain, spinal cord, liver and spleen were stained with hematoxylin-eosin for routine histopathological evaluation. Brain and spinal cord sections were also stained for myelin with luxol fast blue and counterstained with eosin. Coronal sections of brain were prepared from the levels of the hypothalamus and the occipital pole, which include the posterior edge of the hippocampal fissure. Spinal cords were cut in longitudinal sections. For viral antigen detection, immunoperoxidase staining (Vectastain-ABV kit, Vector Laboratories, Burlingame, California) was performed, using MAb J.3.3, specific for the N protein of JHM strain (Fleming *et al*, 1983).

RNA analysis of viruses isolated from the brain

DBT cell cultures were infected with the clarified homogenate from the brains of infected mice, as described above. Culture media were harvested at 24 h p.i. and used to infect additional DBT cell cultures. Supernatants were harvested at 14 h p.i., viral particles purified, and virion genomic RNA extracted as previously described (Makino *et al*, 1984). To determine the copy number of UCUEA repeats in the viral RNA, primer extension studies were performed on the genomic RNA according to the published procedure (Makino *et al*, 1988), using a 5'-end ^{32}P -labeled synthetic oligonucleotide primer #56 (5'-CGCCGAATGGACACGTC-3'), which is complementary to nucleotides 172-188 from the 5'-end of viral RNA.

Primary mixed glial and astrocyte cell cultures

Primary mixed glial cells were prepared from the brains of neonatal C57BL/6 mice (Simonsen Laboratories, Gilroy, California) according to procedures described by McCarthy and de Vellis (1980). Culture medium consisted of DMEM/Hams F12 (1:1; JRH Prioscience, Lenexa, Kansas) supplemented with 10% FCS, 16 mM Hepes, 2.5 mM L-glutamine and 100 mg ml⁻¹ penicillin-streptomycin. Astrocyte-enriched cultures were prepared from 12- to 14-day-old mixed glial cell cultures by mechanical shaking (McCarthy and de Vellis, 1980). Immunofluorescence staining demonstrated that the astrocyte cultures contained >95% cells staining for glial fibrillary acidic protein (GFAP MAB; Boehringer Mannheim, Indianapolis, Indiana), while the mixed glial cultures contained approximately 40-45% GFAP-positive cells, 25-30% galactocerebroside-positive oligodendrocytes (CalC; polyclonal antiserum generously provided by M. Smith, Stanford University, Palo Alto, California), 10-20% A2B5-staining cells (CRL 1520; ATCC, Rockville, Maryland) and 10-15% Mac-1-positive macrophage/microglial cells (TIB 128; ATCC). FITC-

labeled secondary antibodies of the appropriate species specificity were obtained from Jackson Immunoresearch Laboratories (West Grove, Pennsylvania).

Determination of virus growth in primary cell cultures

Astrocyte-enriched and mixed glial cultures were plated in 24-well plates and used for virus infection before day 24 *in vitro*. Cells were infected with JHM(2) or JHM(3) virus at 1×10^6 PFU per well. Culture media and infected cells were harvested from two wells each at 0 h, 12 h, and 1, 2, 3, 4, 6, 8, 10 and 14 days postinfection. Infected cells were harvested by trypsinization and lysed by freezing and thawing three times. Both culture medium and lysed cells were serially diluted, and 0.2 ml of each dilution were inoculated onto confluent monolayers of DBT cells in 60-mm plates. After 1 h adsorption, cells were overlaid with 0.8% agarose containing MEM, 2.5 mM L-glutamine, 100 mg ml⁻¹ penicillin/streptomycin, 5% TPB and 1.5% FCS. Cells were stained with neutral red after overnight incubation and the number of plaques determined.

cDNA cloning and sequencing of S gene from JHM(2) and JHM(3)

The strategy of cloning and sequencing of the S gene of JHM(2) and JHM(3) is summarized in Figure 5. The S gene was cloned into three overlapping

cDNA fragments by reverse transcription (RT)-polymerase chain reaction (PCR) (Makino *et al*, 1988), using primer combinations of 305–306, 307–308 and 309–310, respectively. Briefly, intracellular RNA was isolated from virus-infected cells as described previously (Makino *et al*, 1984), annealed with primers 306, 308 and 310, and reverse-transcribed. The cDNA was amplified by PCR after addition of a second primer, 305, 307 and 309, respectively. The reaction mixtures were subjected to 25 cycles of amplification, consisting of 94°C for 30 s and 72°C for 3 min. After agarose gel purification of these PCR products (approximately 1.3 kb in length), cDNA clones were treated with polynucleotide kinase and T4 DNA polymerase (Boehringer Mannheim Biochemicals), and cloned into Sma I site of vector pTZ18U (United States Biochemical Corp). DNA sequencing was performed by dideoxy chain termination method following the procedure of Sequenase version 2.0 DNA sequence kit (U.S. Biochemical), using primers as indicated in Figure 5.

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References

- Buchmeier MJ, Lewicki HA, Talbot PJ, Knobler RL (1984). Murine hepatitis virus (strain JHM)-induced neurologic disease is modulated *in vivo* by monoclonal antibody. *Virology* **132**: 261–270.
- Collins AR, Knobler RL, Powell H, Buchmeier MJ (1982). Monoclonal antibodies to murine hepatitis virus -4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. *Virology* **119**: 358–371.
- Dalziel RG, Lampert PW, Talbot PJ, Buchmeier MJ (1986). Site-specific alteration of murine hepatitis virus type-4 (MHV-4) peplomer glycoprotein E2 results in reduced neurovirulence. *J Virol* **59**: 463–471.
- Deregt D, Babiuk LA (1987). Monoclonal antibodies to bovine coronavirus: Characteristics and topographical mapping of neutralizing epitopes on the E2 and E3 glycoproteins. *Virology* **161**: 410–420.
- Fazakerley JK, Parker SE, Bloom F, Buchmeier MJ (1992). The V5A13.1 envelope glycoprotein deletion mutant of mouse hepatitis virus type-4 is neuroattenuated by its reduced rate of spread in the central nervous system. *Virology* **187**: 178–188.
- Fleming JO, Stohlman SA, Harmon R, Lai MMC, Frelinger JA, Weiner LP (1983). Antigenic relationship of murine coronaviruses: Analysis using monoclonal antibodies to JHM (MHV-4) virus. *Virology* **131**: 296–307.
- Fleming JO, Trousdale MD, El-Zaatari FAK, Stohlman SA, Weiner LP (1986). Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. *J Virol* **58**: 869–875.
- Fleming JO, Shubin RA, Sussman MA, Casteel N, Stohlman SA (1989). Monoclonal antibodies to the matrix (E1) glycoprotein of mouse hepatitis virus protect mice from encephalitis. *Virology* **168**: 162–167.
- Fleming JO, Wang F-I, Trousdale MD, Hinton DR, Stohlman SA (1993). Interaction of immune and central nervous systems: Contribution of anti-viral Thy 1+ cells to demyelination induced by coronavirus JHM. *Reg Immunol* **5**: 37–43.
- Gallagher TM, Parker SE, Buchmeier MJ (1990). Neutralization-resistant variants of a neurotropic coronavirus are generated by deletions within the amino-terminal half of the spike glycoprotein. *J Virol* **64**: 731–741.
- Hirano N, Fujiwara K, Hino S, Matsumoto M (1974). Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. *Arch Gesamte Virusforsch* **44**: 298–302.
- Knobler RL, Dubois-Dalcq M, Haspel MV, Claysmith SP, Lampert PW, Oldstone MBA (1981). Selective localization of wild type and mutant mouse hepatitis virus (JHM strain) antigens in CNS tissue by fluorescence, light and electron microscopy. *J Neuroimmunol* **1**: 81–92.
- Kubo H, Yamada YK, Taguchi F (1994). Localization of neutralizing epitopes and the receptor-binding site within the amino-terminal 330 amino acids of the murine coronavirus spike protein. *J Virol* **68**: 5403–5410.

- Lai MMC, Brayton PR, Armen RC, Patton CD, Pugh C, Stohlman SA (1981). Mouse hepatitis virus A59: Messenger RNA structure and genetic localization of the sequence divergence from the hepatotropic strain MHV 3. *J Virol* **39**: 823-834.
- Lampert PW, Sims JK, Kniazeff AJ (1973) Mechanism of demyelination in JHM virus encephalomyelitis: Electron microscope studies. *Acta Neuropathol* **24**: 76-85.
- Lee H-J, Shieh C-K, Gorbalenya AE, Koonin EV, La Monica N, Tuler J, Bagdzyahdzhyan A, Lai MM-C (1991). The complete sequence (22 kilobases) of murine coronavirus gene 1 encoding the putative proteases and RNA polymerase. *Virology* **180**: 567-582.
- Leibowitz JL, Weiss SR, Paavola E, Bond CW (1982). Cell-free translation of murine coronavirus RNA. *J Virol* **43**: 905-913.
- Leibowitz JL, Wilhelmsen KC, Bond CW (1981). The virus specific intracellular RNA species of two murine coronaviruses: MHV-A59 and MHV-JHM. *Virology* **114**: 39-51.
- Makino S, Lai MM-C (1989). Evolution of the 5'-end of genomic RNA of murine coronaviruses during passages *in vitro*. *Virology* **169**: 227-232.
- Makino S, Taguchi F, Hirano N, Fujiwara K (1984). Analysis of genomic and intracellular viral RNAs of small plaque mutants of mouse hepatitis virus, JHM strain. *Virology* **139**: 138-151.
- Makino S, Soe LH, Shieh C-K, Lai MMC (1988). Discontinuous transcription of coronavirus mRNAs generates a variable number of pentanucleotide repeats at the leader fusion site. *J Virol* **62**: 3870-3873.
- McCarthy KD, de Vellis J (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissues. *J Cell Biol* **85**: 890-902.
- Nakanaga K, Yamanouchi K, Fujiwara K (1986). Protective effect of monoclonal antibodies on lethal mouse hepatitis virus infection in mice. *J Virol* **59**: 168-171.
- Pachuk CJ, Bredenbeek PJ, Zoltick PW, Spaan WJM, Weiss SR (1989). Molecular cloning of the gene encoding the putative polymerase of mouse hepatitis coronavirus strain A59. *Virology* **171**: 141-148.
- Parker SE, Gallagher TM, Buchmeier MJ (1989). Sequence analysis reveals extensive polymorphism and evidence of deletions within the E2 glycoprotein gene of several strains of murine hepatitis virus. *Virology* **173**: 664-673.
- Pfleiderer M, Routledge E, Herrler G, Siddell SG (1991). High level transient expression of the murine coronavirus haemagglutinin-esterase. *J Gen Virol* **72**: 1309-1315.
- Shieh C-K, Lee H-J, Yokomori K, La Monica N, Makino S, Lai MMC (1989). Identification of a new transcriptional initiation site and the corresponding functional gene 2b in the murine coronavirus RNA genome. *J Virol* **63**: 3729-3736.
- Siddell SG (1983). Coronavirus JHM: Coding assignments of subgenomic mRNAs. *J Gen Virol* **64**: 113-125.
- Sorensen O, Perry D, Dales S (1980). *In vivo* and *in vitro* models of demyelinating diseases. III. JHM virus infection of rats. *Arch Neurol* **37**: 478-484.
- Stohlman SA, Weiner LP (1981). Chronic central nervous systems demyelination in mice after JHM virus infection. *Neurology* **31**: 38-44.
- Stohlman SA, Baric RS, Nelson GN, Soe LH, Welter LM, Deans RJ (1988). Specific interaction between coronavirus leader RNA and nucleocapsid protein. *J Virol* **62**: 4288-4295.
- Storz J, Zhang XM, Rott R (1992). Comparison of hemagglutinating, receptor-destroying and acetyl-esterase activities of avirulent and virulent bovine coronavirus strains. *Arch Virol* **125**: 193-204.
- Sturman LS, Holmes KV, Behnke J (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *J Virol* **33**: 449-462.
- Sturman LS, Ricard CS, Holmes KV (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Activation of cell fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. *J Virol* **56**: 904-911.
- Sundarraj N, Schachner M, Pfeiffer SE (1975). Biochemically differentiated mouse glial lines carrying a nervous system specific cell surface antigen (NS-1). *Proc Natl Acad Sci USA* **72**: 1927-1931.
- Taguchi F, Massa PT, ter Meulen V (1986). Characterization of a variant virus isolated from neural cell culture after infection of a mouse coronavirus JHMV. *Virology* **155**: 267-270.
- Vlasak R, Luytjes W, Lieder J, Spaan W, Palese P (1988a). The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetyltransferase activity. *J Virol* **62**: 4686-4690.
- Vlasak R, Luytjes W, Spaan WJM, Palese P (1988b). Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. *Proc Natl Acad Sci USA* **85**: 4526-4529.
- Wang F-I, Stohlman SA, Fleming JO (1990). Demyelination induced by murine hepatitis virus JHM strain (MHV-4) is immunologically mediated. *J Neuroimmunol* **30**: 31-44.
- Wang F-I, Fleming JO, Lai MMC (1992). Sequence analysis of the spike protein gene of murine coronavirus variants: Study of genetic sites affecting neuropathogenicity. *Virology* **186**: 742-749.
- Wege H, Winter J, Meyermann R (1988). The peplomer protein E2 of coronavirus JHM as a determinant of neurovirulence: Definition of critical epitopes by variant analysis. *J Gen Virol* **69**: 87-98.
- Weiner LP (1973). Pathogenesis of demyelination induced by a mouse hepatitis virus (JHM virus). *Arch Neurol* **18**: 298-303.
- Yokomori K, La Monica N, Makino S, Shieh C-K, Lai MMC (1989). Biosynthesis, structure, and biological activities of envelope protein gp65 of murine coronavirus. *Virology* **173**: 683-691.
- Yokomori K, Banner LR, Lai MMC (1991). Heterogeneity of gene expression of hemagglutinin-esterase (HE) protein of murine coronavirus. *Virology* **183**: 647-657.
- Yokomori K, Baker SC, Stohlman SA, Lai MMC (1992). Hemagglutinin-esterase (HE)-specific monoclonal antibodies alter the neuropathogenicity of mouse hepatitis virus. *J Virol* **66**: 2865-2874.
- Yu X, Bi W, Weiss SR, Leibowitz JL (1994). Mouse hepatitis virus gene 5b protein is a new virion envelope protein. *Virology* **202**: 1018-1023.
- Zhang X, Liao C-L, Lai MMC (1994). Coronavirus leader RNA regulates and initiates subgenomic mRNA transcription, both in trans and in cis. *J Virol* **68**: 4738-4746.