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Demyelinating and nondemyelinating strains of mouse hepatitis virus differ in their neural cell tropism.

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Abstract

Some strains of mouse hepatitis virus (MHV) can induce chronic inflammatory demyelination in mice that mimics certain pathological features of multiple sclerosis (MS). We have examined neural cell tropism of demyelinating and non-demyelinating strains of mouse hepatitis virus in order to determine whether central nervous system (CNS) cell tropism plays a role in demyelination. Previous studies demonstrated that recombinant MHV strains, isogenic other than for the spike gene differ in the extent of neurovirulence and the ability to induce demyelination. Here we demonstrate that these strains also differ in their ability to infect particular cell type(s) in the brain. Furthermore, there is a correlation between the differential localization of viral antigen in spinal cord gray matter versus white matter during acute infection and the ability to induce demyelination later on. Viral antigen from demyelinating strains is detected initially in both gray and white matter, with subsequent localization to white matter of the spinal cord, whereas viral antigen localization of non-demyelinating strains is mainly restricted to gray matter. This suggests that the localization of viral antigen to white matter during the acute stage of infection is essential for induction of chronic demyelination. Overall, these observations suggest that isogenic demyelinating and non-demyelinating strains of MHV, differing in the spike protein expressed, infect neurons versus glial cells in different proportions and that differential tropism to a particular CNS cell type may play a significant role in mediating the onset and mechanisms of demyelination.

Introduction

Multiple sclerosis (MS) is a common disabling neurological disease, pathologically characterized by demyelination, loss of oligodendroglial cells, and axonal degeneration (25, 26). The mechanisms that culminate in the destruction of oligodendrocytes and loss of central nervous system (CNS) myelin are not well understood. The process is believed to involve a T-cell mediated autoimmune phenomenon that may be triggered by one or more viral infections (1). Several experimental viral model systems have been instrumental in providing these insights (2). One of the animal models is based on mouse hepatitis virus (MHV)-induced demyelination in mice that mimics the pathology of MS (12, 19, 21, 39, 42). Infection with neurotropic MHV strains produces a bi-phasic neurological disease with acute meningoencephalitis preceding the onset of chronic demyelination (21).

Following intracranial inoculation (i.c.), MHV is first observed in the brain and subsequently spreads into the spinal cord (30). Viral titer reaches its peak at approximately day 5 post-infection. Infectious viral particles are cleared within the first 7 to 10 days after infection, although viral RNA persists in the white matter of the spinal cord for several months (20, 29). Viral RNA persistence has been demonstrated in spinal cords of mice infected with demyelinating strains of MHV, including MHV-A59 and JHM (17, 20, 29), and has been suggested to be a prerequisite for MHV-induced demyelination. Indeed, MHV-2 and Penn97-1(a non demyelinating recombinant strain of MHV) (3), which do not persist, also do

not cause demyelination (4). Interestingly, the viral RNA of SMHV2-RA59, a chimeric strain (non-demyelinating strain of MHV, expressing MHV-2 spike gene in the MHV-A59 background, previously called Penn 98-1/2) persists in the spinal cord during chronic infection, and yet is unable to induce demyelination (4), indicating that viral RNA persistence in the spinal cord *per se* is insufficient to induce chronic demyelination. Genes for the spike protein, which are known to mediate viral entry, if exchanged between strains may alter the capacity of MHV to infect and replicate in particular CNS cell types (neurons, astrocytes, microglia and oligodendrocytes) during the acute stage of infection, and therefore influence the induction of chronic demyelination.

Little is known about the involvement of CNS cell tropism in MHV-induced demyelination. It is known, however, that in the CNS, demyelinating MHVs infect a variety of neural cell types, including neurons, astrocytes, oligodendrocytes, microglia, and ependymal cells (15, 19, 21, 43). Recently by using JHM spike chimeric viruses, Phillips et al. (32) demonstrated that spike gene-mediated neurovirulence of MHV is associated with extensive viral spread in the brain in both neurons and astrocytes. However, no studies have examined whether non-demyelinating MHV strains infect the same CNS cell types as demyelinating strains.

We have examined CNS cell tropism of both demyelinating and non-demyelinating MHV strains during the acute stage of infection, in order to

determine how the infection of a particular cell type(s) influences the likelihood of development of chronic demyelination. This entailed asking whether non-demyelinating strains differ in their ability to infect gray and white matter cells in the spinal cord during acute infection.

We have used recombinant MHVs: SJHM-RA59$_{EGFP}$ (highly neurovirulent and demyelinating), RA59$_{EGFP}$ (weakly neurovirulent but demyelinating)(38), and SMHV2-RA59$_{EGFP}$ (neurotropic but non-demyelinating)(33). All three viruses express enhanced green fluorescent protein (EGFP) and are isogenic, except for the spike gene, and differ in their neurovirulence and demyelination properties.

During acute infection these three isogenic recombinant MHVs differ in spread and also in their CNS cell tropism. Most interestingly, viral antigen of the demyelinating strains is first detected in gray matter, with subsequent localization to white matter; while antigen of non-demyelinating strains is mainly restricted to gray matter with occasional distribution to white matter. The distribution of viral antigen in white matter during the acute stage of infection may be one of the key factors in chronic viral persistence and induction of demyelination.

Materials and Methods

Viruses and cells: EGFP expressing recombinant viruses SJHM-RA59$_{EGFP}$ and RA59$_{EGFP}$ are isogenic except that SJHM-RA59$_{EGFP}$ expresses the JHM spike gene and RA59$_{EGFP}$ carries the MHV-A59 spike gene (38). These two EGFP expressing

viruses were generated from our previous studies (38) where they were named S_{JHM}-R_{EGFP} and S_{A59}-R_{EGFP} for SJHM-RA59$_{EGFP}$ and RA59$_{EGFP}$, respectively.

Construction of SMHV2-RA59$_{EGFP}$: To construct a non demyelinating EGFP tagged isogenic recombinant strain SMHV2-RA59$_{EGFP}$, the MHV-2 (a non demyelinating MHV strain)(3) spike gene was cleaved from pMH54-SMHV2$_{EGFP}$ with AvrII and SbfI restriction digestion and inserted into the pMH54$_{EGFP}$ in place of the A59 spike gene (4)**.** Targeted RNA recombination was carried out as described in our previous published work (38). For each desired recombinant, at least two strains derived from independent recombination events were characterized. EGFP expressing viruses with the MHV-2 spike gene were labeled as SMHV2-RA59$_{EGFP}$. We previously used this recombinant EGFP tagged strain of SMHV2-RA59 in our other studies (33).

Inoculation of mice: Four-week-old, MHV-free C57Bl/6 (B6) male mice (National Cancer Institute, Frederick, MD) were used in all experiments. Mice were anesthetized with isoflurane (IsoFlo; Abbott Laboratories, North Chicago, IL) prior to inoculation. For intracranial inoculations, 20 µl of diluted virus (in PBS containing 0.75% bovine serum albumin) were injected into the left cerebral hemisphere. Mock-infected controls were inoculated similarly but with an uninfected 17Cl-1 cell lysate at a comparable dilution. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Virulence and viral titers in mice: Virulence was assayed by calculating the lethal dose (LD_{50})**.** Mice were inoculated intracranially with 10-fold serial dilutions of viruses (five mice per dilution). Signs of disease or death were monitored on a

daily basis up to 30 days post infection. $LD_{(50)}$ values were calculated by the Reed-Muench method (34). For measurement of viral titers in the liver and the brain, mice were sacrificed at 1, 3, 5, 7, 9 and 11 days post infection and perfused with 2 ml of sterile PBS. Brains and livers were removed aseptically and separately placed directly into 5 ml of isotonic saline with 0.167% gelatin (gel saline). All organs were weighed and stored frozen at -80 ºC. Brains and livers were homogenized, and viral titers were determined by plaque assay on L2 cell monolayers (4).

Histopathological analysis: For analysis of organ pathology, mice were infected intracranially, sacrificed at various times (day 3, 5, 7 and 10 post infection), and perfused with 5 ml of saline followed by 10% phosphate-buffered formalin. Brains and spinal cords were removed and tissues were embedded in paraffin and sectioned for staining. Brain and spinal cord sections were stained with hematoxylin and eosin (H&E) and used for pathological evaluations by light microscopy. For assessment of demyelination, mice were inoculated intracranially with 0.5 $LD_{(50)}$ dose (8 mice per virus strain) and sacrificed at day 30 post infection. Spinal cord sections were stained with Luxol fast blue to detect plaques of demyelination. We repeated the demyelination experiment five times with 8 mice in each group. Demyelination was quantified by examining four quadrants from two different levels of spinal cord sections for each mouse; thus approximately eighty quadrants were examined for each virus. All slides were coded and read in a blinded fashion (4).

Immunohistochemical analysis: Immunohistochemical analysis was performed by the avidin-biotin-immunoperoxidase technique (Vector Laboratories, Burlington, CA) using 3,3' diaminobenzidine as substrate, and a 1:20 dilution of monoclonal antibody (MAb) directed against the nucleocapsid protein (N) of MHV-JHM (Mab clone 1-16-1, kindly provided by Dr. Julian Leibowitz). Control slides from mock-infected mice were incubated in parallel. All slides were read in a blinded manner.

Immunofluorescence: Mice were inoculated intracranially and sacrificed (5 mice per group) at days 3, 5, 7 post-infection. Infected mice were perfused transcardially with PBS followed by cold PBS containing 4% paraformaldehyde and finally with 10 ml of PBS containing 10% sucrose. Brains were removed and placed at 4 ℃ for 4 h in 10% sucrose and followed by 30% sucrose overnight. Tissues were embedded in OCT medium (Tissue Tek, Hatfield, PA), sectioned sagitally with a microtome to 6 μm thickness and mounted on glass slides. Some sections were stained with H&E while others were left unstained for EGFP detection and immunofluorescence. To detect EGFP fluorescence without immunostaining, frozen brain sections were fixed in ice-cold 95% ethanol for 20 min, incubated at room temperature in ice-cold PBS for 10 min, and then mounted in 4.8% MOWIOL in 50% glycerol. Sections were visualized using an Olympus X-70 microscope system with a 10X long-working distance UPlanF1 phase objective and a filter pack suitable for green fluorescence (U-MWIBABP460-490 DM505 BA515-550). We repeated the acute stage cell

tropism experiment with 5 mice in each group at 0.5 LD_{50} dose and repeated the experiment 3 times. We also infected groups of 5 at 500 PFUs and 1000 PFUs and obtained similar results in terms of cellular tropism. This experiment was repeated twice.

***Double label immunofluorescence**:* Frozen tissue sections were washed with PBS at room temperature in a humidified chamber, incubated for 10 min at room temperature with 1 mg/ml $NaBH_4$ in PBS to reduce auto fluorescence, washed, incubated for 1 h at room temperature with 1 M glycine in PBS to reduce nonspecific cross linking, then washed subsequently with PBS, PBS + 0.5% Triton X-100 (TX) and PBS + TX + 2% goat serum (GS). The sections were incubated overnight at 4ºC with a primary antiserum diluted in PBS+TX+GS, washed and then incubated with a secondary antiserum diluted into PBS+GS for 2 h at room temperature. All incubations were carried out in a humidified chamber. Viral antigen was detected by EGFP in FITC channel (38). Neurons were identified with a 1:50 dilution of mouse monoclonal antibody AP14(10), which recognizes the MAP2b protein (5, 36) (gift from Dr. Virginia M.-Y. Lee, University of Pennsylvania, PA). Astrocytes were identified with a 1:100 dilution of a rabbit anti-cow glial fibrillary acidic protein (GFAP) (Dako Corp**.** Carpinteria, CA). Secondary antibodies were 1:100 dilution of Texas red conjugated goat anti mouse IgG or anti rabbit IgG (Jackson Immune Research, West Grove, PA). Control slides were incubated in parallel with pre-immune rabbit sera, and sections from mock-infected mice were incubated with secondary antibodies

only. Tissue sections were sequentially washed with PBS+TX and PBS, mounted and visualized by fluorescence microscopy with a 40XUPlanApo oil immersion objective, with the iris diaphragm partially closed to limit the contribution of out-of-plane fluorescence, and with filter packs suitable for green fluorescence (U-MWIBA BP460-490 DM505 BA515-550) and red fluorescence (U-MNG BP530-550 DM570 BA590-800+). Images were acquired with a Hamamatsu Orca-1 CCD camera and Image pro image analysis software (Media Cybernetics, Silver Spring, MD).

Results

Comparative pathogenesis of demyelinating and non-demyelinating strains: We have compared the pathogenesis of SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP,}$ which are isogenic except for the spike gene (Fig 1). We used half of an LD $_{(50)}$ (0.5LD$_{50}$) for these pathogenesis studies; this dose for SJHM-RA59$_{EGFP}$ = 5,000 PFUs, for RA59$_{EGFP}$ = 20,000 and SMHV2-RA59$_{EGFP}$=100 PFUs. We infected 8 mice in each group and repeated the experiment 5 times altogether. Out of a total of 40 mice infected for each group, 26 mice survived in the SJHM-RA59$_{EGFP}$ infected group, 30 mice survived in the RA59$_{EGFP}$ infected group and 24 mice survived in the SMHV2-RA59$_{EGFP}$ infected group at day 30 post infection. Brain and spinal cord sections from mice infected intracranially with SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$ were

stained either with H&E or Luxol fast blue and analyzed by light microscopy. No demyelination was observed in any of the SMHV2-RA59$_{EGFP}$ (60% survival rate) infected mice (Fig. 2 Upper Panel), similar to its parental recombinant virus SMHV2-RA59 (previously called Penn 98-1/2)(4). In contrast, demyelination was observed in 100% of the RA59$_{EGFP}$ mice and 60% of the SJHM-RA59$_{EGFP}$ mice (> 70 % survival rate) (Fig. 2, Upper Panel). This confirms our previous findings that the spike gene determines the demyelination property of MHV.

In order to ensure that the non-demyelinating property of SMHV2-RA59$_{EGFP}$ is not due to its inability to replicate in the host, we carried out intracranial inoculations at 0.5

LD $_{(50)}$ dose of two independently isolated clones of SMHV2-RA59$_{EGFP}$ and measured virus replication in mouse brain and liver as a function of time post-infection. The kinetics of viral replication and the final titers of viruses were similar, albeit slightly lower (10-fold), for the EGFP-expressing viruses compared to wild type recombinant virus. The peak of replication was observed at day 5, similar to previous studies (4, 31)(data not shown).

Histopathological studies during acute phase of the infection revealed that all three viruses produced meningoencephalitis. Brain pathology consisted of encephalitis, characterized by parenchymal lymphocytic infiltrates and microglial nodules with focal neuronophagia (Fig 2, Middle Panel). The parenchymal

inflammation was more severe in SJHM-RA59$_{EGFP}$ infected mice brains than in RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$, and brain sections from RA59$_{EGFP}$ showed comparatively more parenchymal inflammation compared to SMHV2-RA59$_{EGFP}$. Associated lymphocytic meningitis was also present in all three strains (Fig 2, Lower Panel). Pathogenic properties of these viruses are summarized in Table 1.

Liver pathology consisted of moderate to severe hepatitis following RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$ infection, similar to the respective parental recombinant strains (28). The infection is characterized by multiple foci of necrosis throughout the liver (data not shown). We did not observe any liver pathology in SJHM-RA59$_{EGFP}$ virus infected mice.

Individual strains exhibit different cellular tropism: To compare the cellular tropism of demyelinating versus non-demyelinating MHV strains, we performed double label immunofluorescence on sagittal brain sections. Mice were inoculated intracranially with 0.5 LD $_{(50)}$ dose of SJHM-RA59$_{EGFP,}$ RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$. Infected animals were sacrificed at days 3 and 5 post-infection and frozen sections were made. EGFP fluorescence was used to detect viral antigen positive cells, and immunofluorescent staining was carried out using MAP2b as a neuron specific marker, and GFAP as an astrocyte specific marker. Sections were systematically scanned in a blinded fashion. Representative pictures of dual color fluorescence of viral antigen plus MAP2b

and viral antigen plus GFAP are shown in Fig. 3 and 4, respectively. Visual microscopic observations show that SJHM-RA59$_{EGFP}$ infects neurons more than the other two strains. On the contrary, in SJHM-RA9$_{EGFP}$ infected mice only a very small number of viral antigen positive cells were also positive for the astrocytic marker GFAP, whereas in RA59$_{EGFP}$ infected mice, EGFP positive cells partially overlapped with GFAP staining. It appears there are other CNS cells which are infected by RA59$_{EGFP}$ that are not astrocytes. More surprisingly, in SMHV2-RA59$_{EGFP}$ infected mice EGFP positive cells were in complete overlap with GFAP, demonstrating that most infected cells were astrocytes. Thus, SJHM-RA59$_{EGFP}$ is predominantly neuron tropic but may be less able to infect astrocytes. RA59$_{EGFP}$ is multitropic and infects neurons as well as astrocytes and also other cell types in the CNS; SMHV2-RA59$_{EGFP}$ infects astrocytes mainly, and neurons with markedly reduced efficiency. To avoid a high percentage of mortality at high virus dose and simultaneously to produce an optimal level of viral replication that does not damage the tissue to a degree that makes microscopic observation unsuitable, we chose a 0.5 LD50 dose. However, to ensure that viral tropism of virus during the acute stage of infection does not depend on the dose, we also performed experiments which demonstrate that the cellular tropism of SMHV2-RA59$_{EGFP}$ at 500 PFUs and 1000 PFUs at day 5 was similar to that at 100 PFU. We also carried out infections with 500 PFUs and 1000 PFUs of SJHM-RA59$_{EGFP}$ and RA59$_{EGFP}$ and observed the same CNS cell tropism as at higher dose, only with fewer infected cells.

Demyelinating and non-demyelinating strains differ in their viral antigen localization in gray and white matter of the spinal cord. To examine whether demyelinating and non-demyelinating strains differ in viral antigen distribution in spinal cord gray and white matter, we collected spinal cord tissues from SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$ infected mice at days 5 and 7 post-infection. Cross sections from several regions of all spinal cords were stained with viral anti nucleocapsid antiserum. 10 mice were sacrificed per time point following infection by a particular viral strain. At day 5 post-infection viral antigens were mainly observed in the gray matter of the spinal cord for all three strains, but in SJHM-RA59$_{EGFP}$ and RA59$_{EGFP}$ infected mice some viral antigen was also detected in the white matter (Fig. 5, Upper Panel). At day 7, most of the viral antigen in SJHM-RA59$_{EGFP}$ and RA59$_{EGFP}$ was found in white matter, whereas SMHV2-RA59$_{EGFP}$ viral antigen remained mainly restricted to gray matter with occasional distribution to white matter (Fig. 5, Middle Panel). In SMHV2-RA59$_{EGFP}$ infected mice, viral antigen was occasionally observed in the white matter (3 of 60 spinal cord sections), and in some spinal cord sections of SMHV2-RA59$_{EGFP}$ viral antigen was detected at the gray- white matter junction as shown in Fig 5, Lower Panel. We repeated the experiment three times with 10 mice in each group and we observed similar viral antigen distribution all three experiments.

Discussion

The current studies highlight the important role of neural cell tropism of viral antigen in MHV-induced demyelination in the CNS. Localization studies showed

that MHV strains that differ in their neurovirulence and ability to demyelinate also differ in their ability to infect particular CNS cell types. SJHM-RA59$_{EGFP}$ infects neurons with greater efficiency, and infection may spread more rapidly than RA59$_{EGFP,}$ while SMHV2-RA59$_{EGFP}$ infects neurons with limited efficiency. This is consistent with previous findings (32) that the degree of neurovirulence is not only dependent on neuronal tropism but also on the number of infected neurons and spread of the infection through neurites. Evidence from closely related strains of neurotropic viruses including HIV, TMEV and reovirus supports the hypothesis that CNS cell tropism and spread in CNS cells play a major role in the distribution and type of CNS lesions (18, 41, 45). Studies with LCMV and HIV also suggest that virus strains that exhibit rapid spread are associated with increased immune-mediated pathology (27, 44). Recently, it has been demonstrated that MHV spread to the spinal cord white matter occurs very rapidly and protection from demyelination can be achieved by inhibiting this viral spread during the acute phase of infection by recruiting high numbers of MHV specific CD8+T cells (23).

Our study highlights the diverse modes of infection that may lead to demyelination. In our study the non-demyelinating strain is less able to infect neurons and spread through neurons; on the other hand, demyelinating strains are highly neuron tropic and spread rapidly through neurites. In contrast, it has been observed that a temperature-sensitive demyelinating mutant of JHM infects mainly non-neuronal cells, having a strong tropism specifically for astrocytes, and causes white matter lesions (11, 16). Similarly, it has been demonstrated that a

monoclonal antibody selected JHM variant, a spike protein mutant, infects the glial cells predominantly in the CNS, causing subacute demyelination (8, 9). Interestingly, the neurotropic and non-demyelinating MHV3 strain, which has an *in vitro* tropism for neurons, ependymal cells, and meningial cells but not for astrocytes and oligodendrocytes, can induce an initial ependymitis, meningitis, and encephalitis in the absence of white matter lesions (40). Our *in vivo* data demonstrate that the highly neurovirulent strain SJHM-RA59$_{EGFP}$ has very little tropism for astrocytes. RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$ infect astrocytes with similar efficiency. As demyelinating and non-demyelinating strains produce similar infections in astrocytes, it appears that astrocytic infection during the acute stage alone may not determine the onset of demyelination. Our observations suggest that astrocyte tropism may be a relevant factor in persistent infection but direct astrocytic infection alone may not be sufficient to induce demyelination.

The development of chronic demyelination involves replication and spread of viral antigen in the spinal cord during the acute stage of infection and the persistence of viral RNA in white matter. This is substantiated by our observations on viral spread of non-demyelinating MHV in the spinal cord, where viral antigen during the acute phase of infection is mainly restricted to gray matter. In contrast, viral antigen from demyelinating strains spreads from gray to white matter during the acute stage of infection. Specifically, after intracranial infection and replication, viral antigens from the demyelinating strains SJHM-

$RA59_{EGFP}$ and $RA59_{EGFP}$ are mostly seen in spinal cord gray matter at day 5 post infection and then in the white matter at day 7 post infection. Our results establish that demyelinating and non-demyelinating strains differ in their viral antigen distribution in gray matter and white matter during acute infection. Viral persistence observed previously in SMHV2-RA59 infected mouse spinal cord during the chronic phase of infection may be due to the presence of viral RNA in gray matter (4). Viral RNA for demyelinating strains is known to persist mainly in the white matter (29). Due to lack of viral RNA persistence in the white matter, essential for the induction of demyelination during chronic infection, SMHV2-RA59 is unable to induce demyelination. It is possible that cell-specific viral RNA persistence is necessary for demyelination. These combined observations reinforce the importance of glial cell infection in the onset of demyelination.

It can also be argued that the spread of viral antigen from neuron to neuron and from neuron to glial cells plays a critical role in the induction of chronic stage demyelination. Although there are no experimental data to demonstrate this, it can be suggested, based on our current neural cell tropism studies, that neuroinvasion by MHV may require entry into the nerve endings, transport to the cell body, replication in the cell body, axonal transport to the synapse and transneuronal viral spread. Similar mechanisms of axonal transport of virus particles have been observed for the alpha herpesviruses, Herpes simplex virus and pseudorabies virus (6, 7). Furthermore, Theiler's murine encephalitis virus (TMEV), a non-enveloped virus that, like MHV, causes chronic demyelination in

mice, is able to traffic from the axon into the surrounding myelin in the absence of cell lysis (37). Thus, by a mechanism involving transneuronal spread, MHV may gain access to synaptically linked neuronal circuits and glial cells. It is not clear however, which type(s) of glial cells must be infected in order to promote the development of MHV-induced chronic demyelinating disease.

Our studies focused mainly on neurons and astrocytes, as they represent the majority of infected cells during the acute stage of MHV infection, but we cannot disregard oligodendrocytes. It has been previously demonstrated that MHV can infect oligodendrocytes (22), but the direct cytolytic effect of MHV on oligodendrocytes is unclear (13, 14). Direct viral infection of oligodendrocytes may contribute to MHV-induced demyelination, as observed in another human demyelinating disease, progressive multifocal leukoencephalopathy (PML), which is caused by JC virus infection of oligodendrocytes (24, 35). Experiments are underway to optimize techniques that will facilitate detection of oligodendrocytes in MHV infected tissues.

In summary, combined assessment of brain and spinal cord infections allows us to infer an indirect role of neuronal cell tropism in demyelination. SJHM-RA59$_{EGFP}$ and RA59$_{EGFP}$ infection can both spread from gray matter to white matter and induce demyelination. The inability of the non-demyelinating strain of MHV, SMHV2-RA59$_{EGFP}$, to spread from gray matter to white matter cells in the spinal cord may be due to its inefficient spread through neurites. Acute stage

inflammation and chronic stage demyelination may be two independent phenomena; however, acute stage neuronal infection and the lack of viral spread from neuronal to glial cells are likely key elements in the induction of demyelination.

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Fig. 1. EGFP tagged isogenic recombinant viruses differ in the spike gene. A schematic diagram of the MHV genome expressing EGFP replacing of gene 4a and part of gene 4b. SJHM-RA59$_{EGFP}$ expressing the JHM spike; RA59$_{EGFP}$ expressing A59 spike gene and SMHV2-RA59$_{EGFP}$ in the background of A59 gene. All three recombinant viruses derived are isogenic, differing only in the spike gene.

Fig. 2. CNS pathology of EGFP expressing MHVs. Upper Panel. Demyelination: Luxol fast blue stained spinal cord sections of mice infected with SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ or SMHV2-RA59$_{EGFP}$, at day 30 post-infection. Thin arrows indicate a region of demyelination, and the arrowheads indicate normal myelinated area of white matter. Original magnifications 20X.

Middle Panel. Encephalitis; microglial nodules: Hematoxylin- and Eosin-stained sections from the basal forebrain of mice infected with EGFP tagged MHVs at day 7 post-infection. Arrows indicate the microglial nodules (microglia with elongated nuclei) and lymphocytes in the vicinity of neurons in mice infected with SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$. Original magnifications 200X.

Lower Panel. Meningitis. Hematoxylin- and eosin-stained sections from the basal forebrain of mice infected with EGFP tagged MHVs at day 7 post-infection:

SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$. In all cases, there is a brisk leptomeningeal lymphocytic inflammatory infiltrate. Original magnifications 200X.

Fig. 3. Identification of EGFP MHV-infected neurons in the brain at day 5 post-infection. Mice were infected intracranially with SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$. Infected mice were sacrificed at day 5 post-infection, and the brains were processed, sectioned and then labeled with anti MAP2b as primary- and with Texas red goat anti-mouse IgG as secondary- antibodies. EGFP fluorescence (green) was used to detect viral antigen- positive cells in the basal forebrain. Red fluorescence shows the corresponding labeling of MAP2b in neurons. Merged images show colocalization of MAP2b and EGFP positive cells. Arrows identify cells double positive for viral antigen and MAP2b. Bar= 250 microns.

Fig. 4. Colocalization of EGFP positive cells with astrocyte marker at day 5 post-infection. Mice were infected intracranially with SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$. Infected mice were sacrificed at day 5 post-infection; brains were processed, sectioned and then immune labeled with anti GFAP (astrocytic marker) antiserum, then stained with Texas red goat anti-mouse IgG as secondary antibodies. EGFP fluorescence was used to detect viral antigen positive cells; red fluorescence was used to detect GFAP in astrocytes. Merged images show colocalization of GFAP and EGFP positive cells. Arrows identify cells double positive for viral antigen and GFAP. Bar= 250 microns.

Fig.5. Viral antigen expression in spinal cord sections of mice infected with EGFP- expressing viruses. Mice were inoculated intracranially with SJHM-RA59$_{EGFP}$, RA59$_{EGFP}$ and SMHV2-RA59$_{EGFP}$. Mice were sacrificed at days 5 and 7 post-infection, spinal cord sections were stained with anti nucleocapsid antiserum. Representative hemi-sections and complete sections of spinal cords are shown from the cervical region of the spinal cord of day 5 (**Upper Panel**) and day 7 (**Middle Panel**) infected mice. In SMHV2-RA59$_{EGFP}$ infected mice occasional viral antigen distribution in the white matter and gray-white matter junction is shown in **Lower Panel**.

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