Spread of a Neurotropic Coronavirus to Spinal Cord White Matter via Neurons and Astrocytes

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Received 5 July 1994/Accepted 21 October 1994

Mouse hepatitis virus strain JHM (MHV-JHM) causes a chronic encephalomyelitis in susceptible mice, with histological evidence of demyelination in the spinal cord. After intranasal inoculation, virus spreads retrogradely to several brain structures along neuroanatomic projections to the main olfactory bulb. In the absence of experimental intervention, mice become moribund before the spinal cord is infected. In this study, infusions of anti-MHV neutralizing monoclonal antibodies were administered to protect mice from the MHV-JHM-induced acute encephalitis and to allow survival until virus spread to the spinal cord. Under these conditions, virus was observed to enter specific layers (primarily laminae V to VII) in the gray matter of the upper spinal cord, consistent with transneuronal spread. While the brain structures which are the sources for virus spread to the spinal cord cannot be determined with certainty, the ventral reticular nucleus is likely to be important since it is consistently and extensively labeled in all mice and receives projections from subsequently infected areas of the spinal cord. After initial entry into the gray matter, virus rapidly spread to the white matter of the spinal cord. During the early stages of this process, extensive infection of astrocytes was noted, suggesting that cell-to-cell spread via these glial cells is an important part of this process. Reports from other laboratories using cultured cells strongly suggested that astrocytes serve as important regulators of oligodendrocyte function and, by extrapolation, have a major role in vivo in the processes of both demyelination and remyelination. Thus, our results not only outline the probable pathway used by MHV-JHM to infect the white matter of the spinal cord but also, with the assumption that infection of astrocytes leads to subsequent dysfunction, raise the possibility that infection of these cells contributes to the demyelinating process.

Mouse hepatitis virus (MHV), a member of the coronavirus family, causes encephalomyelitis, gastroenteritis, and hepatitis in susceptible strains of mice and rats (9). In particular, the JHM strain of MHV (MHV-JHM) causes acute encephalitis and acute and chronic demyelinating diseases in these animals (30). The MHV-JHM-induced demyelinating disease is considered a good model for the human disease multiple sclerosis.

After intranasal inoculation, most strains of mice develop an acute, rapidly fatal encephalitis, characterized by widespread infection of neurons (51, 65). Virus enters the central nervous system (CNS) via the olfactory nerve and then spreads transneurally to infect parts of the brain which are neuroanatomic connections of the main olfactory bulb (MOB) (1, 21, 32). In a previous study, we identified in detail the CNS structures infected in 6-week-old C57BL/6 (B6) mice after inoculation into the MOB (1). The infected sites were all well-described connections of the MOB, although not every brain structure connected to the MOB was infected by MHV. Virus infection was consistent with retrograde spread, with little evidence of spread in an anterograde direction. In mice with chronic demyelinating encephalomyelitis, lesions in the spinal cord are the most consistent and prominent pathological feature (31, 42, 56, 58, 65). The mice studied in our previous investigation developed acute encephalitis and were moribund before virus was able to spread to the spinal cord. Thus, it was difficult to determine the precise pathway used by the virus to infect the spinal cord, although viral spread throughout the brain proper appeared to be the same in mice with the acute

encephalitis and in those which later developed the demyelinating disease (1a).

Several approaches are available to decrease the extent of viral spread in the brains of infected mice and thus facilitate spread of virus to the spinal cord. First, mice infected with attenuated strains of MHV do not succumb to the acute encephalitis but rather develop chronic demyelinating encephalomyelitis (10, 17, 23). In several cases, it has been shown that the same structures are involved as in mice infected with virulent MHV-JHM, but less contiguous spread occurs at each site (15, 32). In a second approach, the infection can be diminished by infusion of antiviral antibody or T cells or by nursing with dams previously immunized against the virus (5, 16, 28, 34, 43, 51, 57, 67). In these mice, virus and demyelinating lesions can be detected in the spinal cord at later times post inoculation (p.i.), showing that these experimental manipulations do not prevent virus spread to this structure. However, the precise routes of spread to the spinal cord were not delineated in these studies.

In this study, 3-week-old B6 mice were infected intranasally with MHV-JHM and protected from acute encephalitis with infusions of neutralizing monoclonal antibodies directed against the surface (S) glycoprotein. The amount of antibody was titrated so that virus was not cleared to undetectable levels but was sufficiently diminished in quantity to allow transneuronal passage from the MOB to the spinal cord. Using this model, we showed that virus first appeared in the spinal cord in gray matter, consistent with transneuronal spread from the brain. In addition, this model was also used to analyze virus spread within the spinal cord. Murine astrocytes are readily infected by MHV (33, 50, 60), and the data suggest that astrocytes are an important part of the pathway by which virus spreads from the gray to the white matter within the cord.

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MATERIALS AND METHODS

Animals. Pathogen-free 3-week-old B6 mice were purchased from either Jackson Laboratory (Bar Harbor, Maine), Sasco Laboratories (Omaha, Neb.), or Harlan Laboratories (Indianapolis, Ind.). A total of 68 mice were used in this study. Forty mice were used to determine the pathway of spread to the gray matter of the spinal cord, 23 mice were used to study the colocalization of the astrocyte-specific marker glial fibrillary acidic protein (GFAP) and MHV-JHM, and 5 mice were used for histological studies of long-term survivors.

Virus. MHV-JHM was grown and titered as previously described (51). Mice were protected from acute encephalitis as follows. Mice were intranasally inoculated with 5×10^4 PFU of MHV-JHM under methoxyflurane (Pitman-Moore, Inc., Washington Crossing, N.J.) anesthesia. After 72 h, mice received an intraperitoneal inoculation of 7 μ l of two neutralizing antibodies diluted to 0.5 ml in phosphate-buffered saline. The 7 μ l consisted of 3.5 μ l each of murine monoclonal antibodies 5A13.5 (neutralizing titer, 1:45,000) and 5B19.2 (neutralizing titer, 1:2,700), kindly provided by M. Buchmeier (The Scripps Research Institute). Mice were harvested at 5, 6, and 7 days p.i. and processed as previously described (48).

Histology. Mice were perfused with phosphate-buffered saline, fixed in formalin, and embedded in paraffin. Five-micrometer sections were cut, processed, and stained with luxol fast blue in order to detect areas of demyelination.

In situ hybridization. In situ hybridization using an antisense riboprobe was performed as previously described (46, 48). Positive (mice dying from acute encephalitis) and negative (uninfected mice) brains were included in some experiments. After processing, samples were exposed to X-ray film (Kodak, Rochester, N.Y.) for 2 to 4 days and dipped in NTB-2 emulsion (Kodak) for 2 weeks. Slides were stained with cresyl violet and examined by bright-field and dark-field light microscopy.

Combined immunohistochemistry and in situ hybridization. Brains and spinal cords were harvested at 6 to 7 days p.i. Coronal sections (25 μ m) were cut with a cryostat through the entire spinal cord and caudal medulla at 100- to 200- μ m intervals and placed on silane-treated slides. The sections were fixed with periodate-lysine-paraformaldehyde (38) and treated with 4% normal goat serum for 1 h. After washing, slides were incubated with a 1:500 dilution of rabbit anti-GFAP (kindly provided by L. Eng, Stanford University) overnight at 4°C. The antigen-antibody complex was visualized by using horseradish peroxidase-conjugated goat anti-rabbit antibody (Organon Teknika Corp., Durham, N.C.) with 3,3'-diaminobenzidine as the final substrate. RNasin (50 U/ml; Promega, Madison, Wis.) and heparin (5 mg/ml) were used to inhibit RNase activity during steps containing serum products. Following immunohistochemistry for GFAP, in situ hybridization was performed as described above to detect MHV RNA. Sections were examined for cells labeled by both GFAP immunoreactivity and silver grains. No astrocyte-specific labeling occurred in the absence of primary anti-GFAP antibody or when an irrelevant rabbit antibody was substituted for the specific antibody.

Combined immunoperoxidase and immunofluorescence. Brains and spinal cords were removed from MHV-JHM-infected 3-week-old B6 mice as described above. Cryostat sections were cut through the cervical spinal cord and placed on silane-treated slides. After treatment with 4% normal goat serum for 1 h, slides were incubated overnight with a mixture of mouse monoclonal anti-MHV-JHM antibody (anti-nucleocapsid [N] antibody 5B188.2, kindly provided by M. Buchmeier) and rabbit anti-GFAP antibody. Samples were then treated with horseradish peroxidase-conjugated goat anti-rabbit antibody and developed as described above. The samples were washed and then treated with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (1:100). Samples were air dried and mounted and then examined and photographed by using an Olympus BH-2 microscope with a fluorescent attachment.

RESULTS

Low levels of neutralizing monoclonal antibody attenuate the MHV-JHM infection. In our previous study, the transneuronal spread of MHV-JHM was analyzed in untreated mice (1). Under these conditions, mice infected with virulent strains of MHV become clinically ill and die before virus is able to infect the spinal cord. Buchmeier et al. showed that treatment with a single intraperitoneal inoculation of neutralizing antibody directed against the surface glycoprotein (S) of MHV-JHM protected mice from acute encephalitis, but mice still developed demyelinating disease (5). Thus, virus was able to spread to the white matter in these mice, even in the presence of antiviral antibody. In preliminary experiments, however, we found that intraperitoneal administration of the same volume (0.2 ml) of a 1:1 mixture of two anti-S neutralizing antibodies (5A13.5 and 5B19.2, kindly provided by M. Buchmeier, The Scripps Research Institute) either before or 24 h after intranasal inoculation with MHV-JHM afforded such complete pro-

tection from the acute encephalitis that we were unable to detect any viral RNA in the brain by in situ hybridization analysis even at early times after infection. The greater sensitivity which we observed to antibody administration than was previously reported was most likely due to the fact that virus was administered by intracerebral administration in the prior study whereas intranasal inoculation was used in this study. We performed additional experiments in which we progressively decreased the amount of antibody administered and increased the interval between inoculation of virus and delivery of antibody until mice were still protected from the acute encephalitis, but detectable levels of viral RNA were present in the CNS. We determined that 7 μ l of the antibody mixture administered 72 h after intranasal inoculation of virus provided the desired amount of protection. Mice protected with this amount of antibody developed symptoms consistent with mild signs of encephalitis, including hunching and ruffled fur, at 6 to 7 days p.i. Of the mice harvested at this time, 42 of 47 (89%) showed these symptoms. By day 8, only 1 of 13 was still symptomatic, and of the 7 mice analyzed at this time, 4 were positive for viral RNA when analyzed by in situ hybridization. Since MHV-JHM has the propensity to persist and cause demyelinating lesions in infected mice, we next assayed five of the six long-term survivors for the presence of demyelinating lesions at 2 months p.i. These six mice were completely asymptomatic after the first few days after intranasal inoculation, and none of them developed any clinical symptoms. Evidence of demyelination could be detected in three of five mice examined by routine histological methods (data not shown). The presence of such lesions in these mice shows that virus spread to the spinal cord was not prevented by the presence of neutralizing antibody, in agreement with previous results (5).

In the first set of experiments, we determined whether the same CNS structures were infected in the presence of antibody as in its absence. At 72 h after intranasal inoculation, before the administration of antibody, virus could be detected in significant quantities in the MOB (Fig. 1A), in agreement with previous results (47). In the absence of antibody, virus then spread by 5 to 6 days p.i. to many brain structures neuroanatomically connected to the MOB (1). The presence of neutralizing antibody did not change the pattern of virus spread. It did, however, greatly decrease the lateral spread noted at each site of infection at late times during the acute encephalitis and thus increased the specificity of the viral labeling. As illustrated in Fig. 1, several sites previously reported to be infected after intranasal inoculation with MHV were also labeled in the presence of neutralizing antibody. These sites included the anterior olfactory nucleus (Fig. 1B), the primary olfactory cortex (Fig. 1B), the diagonal band of Broca (Fig. 1C), the ventral pallidum (Fig. 1C), the ventral tegmental area (Fig. 1D), the lateral hypothalamus (Fig. 2A), the medial parabrachial nucleus, and the raphe nuclei.

Appearance of MHV-JHM in the gray matter of the spinal cord. MHV-JHM could conceivably infect the spinal cord via one of several routes, including (i) transneuronal, presumably transsynaptic spread, (ii) glial spread, (iii) hematogenous spread, and (iv) spread via cerebrospinal fluid. From previous analyses of virus spread within the brain, we anticipated that transneuronal spread would be the most important mechanism for spread from the brain to the spinal cord. To determine the site of entry of MHV-JHM into the spinal cord, coronal sections of the brains and spinal cords of 56 mice were analyzed at 5 to 7 days p.i. No spinal cord labeling was detected before day 5. In all other cases, the upper cervical cord was most heavily infected, and extensive labeling was also observed in the caudal medulla adjacent to the upper cord (Fig. 2E and 3A). Virus

FIG. 1. Presence of neutralizing antibodies does not change the pattern of brain structures infected by MHV-JHM. Three-week-old B6 mice were infected intranasally with MHV-JHM and protected from acute encephalitis by intraperitoneal administration of neutralizing monoclonal antibodies directed against the S protein. Brains were harvested and analyzed by in situ hybridization as described in Materials and Methods. (A) Olfactory bulb infection at 72 h p.i., prior to administration of protective antibody. After administration of antibody, the same brain structures were labeled at 5 days p.i. as in the absence of antibody (1), including the anterior olfactory nucleus and primary olfactory cortex (B), the diagonal band of Broca (C), and the ventral tegmental area (D). Magnification bars: A, 1250 μ m; B to D, 500 μ m.

was present only or predominantly in the gray matter of the spinal cord in six of nine mice examined at 5 days p.i. (Fig. 3B). In none of 9 mice at 5 days and 16 of 25 mice at 6 days, virus was present in contiguous areas of the gray and white matter, whereas in 18 of 22 mice at 7 days p.i., MHV could be detected predominantly in the white matter, with a small amount of residual gray matter labeling. By day 8 p.i., virus was cleared from three of seven mice, with the others showing less labeling than was observed in the mice harvested at day 7, although the pattern was similar. In those mice examined at days 6 to 8 days p.i. in which MHV had spread to the white matter, labeling was primarily in the ventrolateral part of the spinal cord (Fig. 3C and D). In a previous report, we showed that virus could be detected only in the ventrolateral white matter of the spinal cord at 15 days p.i. (49). The pattern of labeling which we observed together with these previous results suggest that virus enters the gray matter of the spinal cord by transneuronal spread and then spreads to the white matter.

In general, virus first appeared at specific sites in the gray matter, corresponding to laminae V to VII of the cervical cord (Fig. 3B). Since MHV-JHM spreads within the CNS primarily if not solely from axonal terminals to cell bodies (retrograde direction) and only a limited number of sites in the brain receive projections from the spinal cord, it should be possible to determine the source of the virus which infected the cord. Projections of the spinal cord to several structures in the brain, including the spinoreticular, spinothalamic, spinohypothalamic, spinomesencephalic, and spinocerebellar tracts, have been described (2, 6, 8, 22, 37, 53, 61, 68). None of these tracts originates solely in the distribution of sites in the spinal cord which are infected by MHV-JHM, although some of these projections arise predominantly from the appropriate areas of the cervical spinal cord.

We next determined whether MHV-JHM infected the sites of termination of the aforementioned tracts, including the pontine reticular formation, ventral, dorsal, and lateral medullary reticular nuclei (spinoreticular), the thalamic nuclei (spinothalamic), the medial and lateral hypothalamus (spinohypothalamic), midbrain structures, particularly the periaqueductal gray (spinomesencephalic), the anterior and posterior lobes of the cerebellum, and the medial, the anterior interpositus, and the posterior interpositus cerebellar nuclei (spinocerebellar tract) (Fig. 2). Thalamic labeling was present mainly in the midline nuclei, such as the paraventricular, medial habenula, and submedial nuclei (Fig. 2A). In the hypothalamus, the posterior lateral hypothalamus was heavily infected (Fig. 2A). Labeling was also detected in the mesencephalon in the periaqueductal gray (Fig. 2B). Infection of the pontine and medullary reticular formation appeared on day 5, just prior to infection of the gray matter of the spinal cord (Fig. 2C to E). Heaviest labeling was observed in the pontine nucleus caudalis and ventralis and the medullary gigantocellularis reticular nucleus (Fig. 2C and D). In some cases, labeling was also detected in the dorsal and lateral reticular nuclei (Fig. 2D and

FIG. 2. Infected brain sites receiving projections from the spinal cord. Mice were harvested at 5 days p.i., and brains were analyzed by in situ hybridization. Sites which receive projections from the spinal cord and were infected with MHV-JHM: (A) midline thalamic nuclei (solid arrow), lateral hypothalamus (open arrow), and subthalamic nucleus (arrowhead) (spinothalamic and spinohypothalamic tracts); (B) periaqueductal gray (arrow) (spinomesencephalic tract); (C) pontine reticular nuclei; (D) gigantocellularis (arrowhead) and lateral medullary reticular nuclei (arrow); (E) ventral reticular nuclei in the caudal medulla. Panels C to E are termination sites for the spinoreticular tract. Magnification bar, 500 μ m.

3A). With the exception of the cerebellar structures, MHV-JHM could be detected in one or more of the projection sites associated with each of the aforementioned tracts, and thus these experiments do not definitively show which spinal tract was used by virus to spread to the spinal cord. However, as discussed below, we think it likely that the spinoreticular tract has a major role in this process.

Role of astrocytes in spread from the gray to white matter of the spinal cord. In previous studies of mice with MHV-JHM-induced demyelination, infection of oligodendrocytes has been documented and is believed to be an important part of the pathological process (5, 26, 31). As shown in Fig. 3B to D, after

initial entry into the gray matter of the spinal cord, virus rapidly spreads to infect the white matter of the cord. Since this process occurs in the presence of neutralizing antibody, it is unlikely to result from release of free virus from infected neurons and widespread diffusion into the white matter. More likely, virus infects cells in the white matter from either infected axons or astrocytes with minimal exposure to extracellular fluid. Astrocytes are present in both the gray and the white matter, are in intimate contact with neuronal synapses, and are readily infected by MHV-JHM (13, 36, 50, 60, 66). To determine the role of astrocytes in the spread of MHV-JHM, spinal cords from mice infected 7 days previously were ana-

FIG. 3. Detection of MHV in the dorsal reticular nucleus and in the gray and white matter of the spinal cord. Mice were harvested at 5 to 7 days p.i., and brains and spinal cords were analyzed by in situ hybridization. (A) Presence of virus in the dorsal medullary reticular nucleus at day 5 p.i. (B) Virus was detected only in the gray matter of the cord, primarily in laminae V to VII, in 6 of 9 mice examined at day 5 and 7 of 25 mice examined at day 6. (C and D) Virus spread to the white matter is observed in 16 of 25 mice examined at day 6 and 18 of 22 mice examined at day 7. The approximate positions of laminae I to X are indicated in panel B (3). Magnification bar, 500 μ m.

lyzed for the presence of both virus and GFAP, a protein specific for astrocytes. GFAP is up-regulated as part of the inflammatory response to the virus and is thus easier to detect in infected than in uninfected brains (14, 24).

In the first set of analyses, GFAP was detected by immunohistochemistry and MHV-JHM was detected by in situ hybridization (Fig. 4). As evident from Fig. 4, astrocytes were heavily infected during the initial stages of virus spread from the gray to the white matter of the spinal cord. Infection of astrocytes was most obvious in the white matter. While infected astrocytes were also present in the gray matter, the actual number of double-labeled cells was much smaller in this part of the spinal cord. In some fields, virtually all of the infected cells in the white matter appeared to express GFAP (Fig. 4A and B). Silver grains indicative of viral infection were present both over cell bodies and over the long, darkly stained processes which are characteristic of reactive astrocytes. In some cases (Fig. 4C and D), the virus appears to infect astrocyte cell bodies and processes that extend from the gray to the white matter. The figure also clearly shows that not all astrocytes are infected

with MHV-JHM and that some infected cells do not express GFAP.

To confirm that astrocytes were heavily infected by MHV-JHM, we showed by a second technique that GFAP and MHV-JHM antigens colocalized in the same cells. For these analyses, astrocytes were detected by immunohistochemical techniques as before, but MHV-JHM-infected cells were identified by an indirect immunofluorescence assay using an antibody which recognizes the nucleocapsid protein as described in Materials and Methods. As shown in Fig. 5, several cells were positive for both GFAP and MHV-JHM with this methodology, indicating that many of the infected cells in the white matter were astrocytes and thereby confirming the data presented in Fig. 4.

DISCUSSION

Although the pathogenesis of the demyelinating disease caused by MHV-JHM remains controversial, the data from several studies are most consistent with a model in which virus infection of oligodendrocytes and subsequent host immune

FIG. 4. Spread from the gray matter to the white matter of spinal cord occurs via astrocytes. Spinal cords were harvested from MHV-infected mice at 7 days p.i. and simultaneously assayed for the astrocyte-specific protein GFAP by using immunohistochemistry and for MHV-JHM by using in situ hybridization. Many cells positive for both GFAP (brown product) and MHV-JHM (silver grains) are clearly visible in the illustrations shown. (A) Low-power view illustrating the large number of astrocytes infected by MHV-JHM; (B) part of panel A at higher magnification and rotated 90°. The closed arrows indicate the same infected cells and the open arrows indicate the same uninfected astrocytes in both panels. In panels C and D, MHV appears to infect astrocyte cell bodies and processes that connect the gray matter and the periphery in infected mice. Magnification bars: A, 100 μ m; B to D, 50 μ m.

response lead to the observed pathological changes (27, 54, 62, 65). The relative importance of each in the process remains to be determined, but with the exception of one study (64), all previous results suggest that virus must be present for demyelination to occur. After intranasal inoculation, we and others have shown that MHV spreads transneuronally along synaptically linked neural pathways within the brain (1, 21, 32). It has also been shown that virus is present in the white matter of spinal cord both in asymptomatic mice at 15 days after infection and in mice with demyelinating encephalomyelitis (49). The present study provides information about how virus actually spreads to the spinal cord from the brain and how it moves to infect the white matter.

The demyelinating model used in this study is a variation on the one which we have used previously and is based on earlier studies performed by Buchmeier et al. (5). Previously, we studied mice infected intranasally with MHV-JHM and nursed by dams previously immunized against the virus. These mice are fully protected against acute encephalitis, but a variable fraction (40 to 90%) develop hindlimb paralysis with histological evidence of demyelination 3 to 8 weeks later (51). This model is less useful for the type of study described in this report, since the amount of maternal antibody delivered to each suckling mouse depends on variables which are not easily controlled. Infusion of monoclonal antibodies, as described in this report, is more useful for studies analyzing the pattern of virus spread since the amount of antibody received by each animal can be

tightly controlled and the rate and extent of spread in each animal are consistent. The disadvantage of protecting mice with an infusion of monoclonal antibodies is that mice with demyelinating lesions do not show symptoms. The reason for this lack of symptoms is not known, although demyelinating lesions appear to be smaller in mice protected with monoclonal antibodies than in mice with maternal antibody (unpublished observations). Virus spreads along the same pathways either in the absence or in the presence of either neutralizing monoclonal antibody (Fig. 1) or maternal antibody (data not shown). The net result of the experimental intervention is that the animals survive longer, thus allowing virus to enter the spinal cord and spread to the white matter.

Consistent with previous observations on the transneuronal spread of MHV-JHM, virus was first detected in the gray matter of the spinal cord. In another study using intracranial inoculation of virus, MHV spread to the spinal cord via the cerebrospinal fluid (63). In our study, the distribution of infected cells was different from what was observed after intracranial inoculation, and labeling around the central canal was not observed. This finding suggests that after intranasal inoculation, virus did not spread to the spinal cord via the cerebrospinal fluid to a significant extent. In addition, virus was not detected in a perivascular distribution, suggesting that MHV did not spread hematogenously to the spinal cord. Finally, spread via glial cells was unlikely since the rapidity with which

FIG. 5. Astrocyte infection shown using simultaneous detection of GFAP and MHV antigen. Infected spinal cords were analyzed as described in Materials and Methods for GFAP (immunohistochemistry) (A and C) and MHV (immunofluorescence) (B and D). Several cells positive for both labels are indicated by arrows. Magnification bar, 50 μ m.

virus spread to the spinal cord was most consistent with transneuronal spread.

While the pattern of infection was not the same in all mice, laminae V to VII were the most prominent sites of labeling in all animals. To determine the source for this virus, we assumed that virus spread primarily in a retrograde direction, as described in our earlier study (1), although a recent report analyzing virus egress in neuronal cultures suggested that MHV could also spread anterogradely (45). However, since there is little evidence for anterograde spread within the CNS, we have restricted our analyses to tracts originating in the spinal cord (i.e., retrograde spread). Tracts to the thalamus, mesencephalon, hypothalamus, and pontine and medullary reticular formation are believed to convey somatosensory information from the spinal cord, and all originate to some extent from sites throughout the cervical, thoracic, lumbar, and sacral segments of the cord (4, 6, 8, 22, 53, 59, 61, 68). In addition, most of the laminae of the cord provide projections to each of the aforementioned structures, although the proportion is different for each of the termination sites. An additional difficulty with trying to determine the precise source for viral spread to the spinal cord is that the upper cord also has projections, albeit smaller, to other areas of the telencephalon, including the diagonal band of Broca and ventral pallidum, both heavily infected by MHV-JHM (8). Finally, all of these projections have been defined by inoculation of tracers into specific sites in the brain and spinal cord. Their definition is thus limited by the precision of the original inoculation and the ability of the

injected compounds to diffuse from the site of injection equally to (or from) all levels of the spinal cord.

Even with these caveats, some differences are apparent among the tracts projecting to the above regions of the brain. First, the spinothalamic, spinomesencephalic, and spinoreticular tracts originate to a large extent from the upper cervical cord, with the spinothalamic and spinoreticular tracts showing the greatest predominance of cervical origin (22, 61, 68). The spinal connection to the lateral reticular nucleus is believed to involve the largest number of cells in the spinal cord (53), but a large fraction of these cells are in the lumbar region, an area not heavily infected in our studies. Second, both the spinal connections to the midline thalamic nuclei, which are infected by MHV-JHM (Fig. 2A), and the reticular nuclei originate to a large extent from laminae V to VII in the spinal cord (20, 59, 61). However, the lack of intensity of the labeling in the specific thalamic nuclei compared with that observed in the reticular nuclei makes it less likely that the former are the source of the virus reaching the spinal cord. In contrast, the ventral reticular nuclei, which were consistently and heavily infected by MHV-JHM, receive predominant projections from neurons in laminae V to VII of the cervical spinal cord (61). In addition, infection of the reticular nuclei occurs either prior to or concomitant with infection of the upper spinal cord. Thus, the specificity of the infection in the spinal cord and the pontine and medullary reticular nuclei, the well-documented and specific connections between these two structures, and the tem-

poral course of the MHV-JHM infection all strongly suggest that these nuclei are the source for the spinal cord infection.

After infection of the gray matter, virus spreads to the white matter, with infection of astrocytes as a major component of this process. Not all infected cells are GFAP positive, and so other cells, such as microglia, could conceivably play a role in the spread of MHV-JHM to the white matter. Virus most likely spreads from neurons to astrocytes. This process is probably facilitated by the close physical proximity and intimate physiological relationship between these two types of cells. Astrocytes are located in close juxtaposition to neuronal synapses in the gray matter, are involved in achieving ionic and neurotransmitter homeostasis, and provide trophic support for neurons (14, 25). Recent experiments also suggest that astrocytes and neurons communicate reciprocally through nonsynaptic mechanisms (41, 44).

White matter astrocytes have characteristic long processes which connect the gray and white matter and cross the white matter. As shown in Fig. 5, these processes, or more likely the cell bodies associated with them, are heavily infected by MHV. Gap junctional coupling between astrocytes has been documented by both electrical and dye coupling measurements (11). Astrocyte-astrocyte contact is so extensive that these cells have been postulated to form a generalized functional syncytium which extends large distances within the CNS (12, 40). Gap junctions are probably too small to allow direct passage of virus, but by decreasing the gap between adjacent cellular membranes, they may facilitate MHV-JHM spread to uninfected cells without exposure to the extracellular fluid. Such spread would be furthered by the fusogenic properties of the surface glycoprotein of MHV-JHM; this protein can cause cell fusion and subsequent viral infection even in the absence of specific viral receptor (18).

Oligodendrocytes are infected in mice with acute demyelination, although the role of infection of oligodendrocytes in mice with chronic demyelination is more controversial (29). Infection of oligodendrocytes may occur after virus has spread to the white matter via astrocytes. Astrocytes and oligodendrocytes are also coupled via gap junctions, although these junctions show only weak electrical coupling compared with astrocyte-astrocyte interactions (19). In addition, astrocytes secrete factors which are important for oligodendrocyte differentiation, proliferation, and survival (19). Astrocyte dysfunction, such as might be predicted to occur if substantial numbers of these cells were infected, could affect oligodendrocyte function, with the result being demyelination. In addition, activated astrocytes are known to express both tumor necrosis factor alpha and nitric oxide synthase mRNA (7, 35, 55). Oligodendrocytes are very sensitive to both tumor necrosis factor alpha and nitric oxide (39, 52), and their release by astrocytes might contribute to the demyelinating process. Thus, not only might astrocytes serve as the conduit for passage of virus to the white matter, but infection of these cells could contribute indirectly to the demyelinating process either via the lack of production of necessary growth factors or by synthesis of substances toxic to oligodendrocytes.

ACKNOWLEDGMENTS

We thank C. M. Stoltzfus, E. Barnett, and S. Murphy for critical reading of the manuscript and Paul Reimann for photographic assistance.

This research was supported by NIH grants NS24401 and DC01711. N.S. was supported by NIH training grant T32AI07343, and S.P. was supported by a Research Career Development Award from the NIH.

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