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SHORT COMMUNICATIONS

Spread of a Neurotropic Murine Coronavirus into the CNS via the Trigeminal and Olfactory Nerves

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The route of entry into the central nervous system (CNS) of most neurotropic viruses has not been established. The coronavirus, mouse hepatitis virus strain JHM (MHV-JHM), causes acute encephalomyelitis and acute and chronic demyelinating diseases and is an important model system for virus-induced neurological disease. Suckling C57BL/6 mice infected intranasally with MHV-JHM develop either the acute encephalomyelitis or a late onset, symptomatic demyelinating encephalomyelitis, depending on whether they are nursed by unimmunized or immunized dams. Analysis by *in situ* hybridization was used to determine the route of entry of MHV-JHM into the CNS in these mice. At early times, viral RNA was detected only in the trigeminal and olfactory nerves and in their immediate connections in all mice. A few days later, MHV-JHM RNA was found throughout the brain in mice dying of the acute encephalomyelitis, but remained confined to the entry sites in mice which did not develop acute disease. These results suggest that MHV-JHM enters the CNS via an interneuronal route in all mice, but that the presence of maternal antibody prevents the dissemination of virus via extracellular fluid. In addition, MHV-JHM may establish low-level persistence in the trigeminal or olfactory nerve or in one of its connections in mice that do not develop acute encephalomyelitis. © 1989 Academic Press, Inc.

Coronaviruses, positive-stranded RNA viruses, are able to infect persistently many types of animals (*1, 2*). Neurotropic strains of the murine coronavirus, mouse hepatitis virus (MHV), cause acute encephalomyelitis and acute and chronic demyelinating diseases in susceptible rodents (*2–10*). For this reason, mice or rats infected with these viruses are useful for determining the factors important for both acute and persistent CNS infections caused by RNA viruses. One such factor is the relationship between the initial site of viral entry and the sites of preferential replication in animals that become clinically ill either acutely or after a latent period of several weeks.

Young mice develop an invariably fatal acute encephalomyelitis if inoculated with the appropriate dose of MHV-JHM. Acute disease can be prevented by the use of mutant virus (either temperature sensitive or neutralization resistant), by administration of neutralizing monoclonal antibody prior to inoculation, or by suckling by immunized dams (*11–16*).

Suckling C57BL/6 mice infected intranasally with the JHM strain of MHV and nursed by immunized dams remain asymptomatic for several weeks post inoculation (p.i.), although 40–90% eventually develop a neurological disease characterized by hindlimb paralysis. Signs of encephalitis (hunching, ruffled fur, irritability) are not present at this time. MHV-JHM RNA can be detected in the brainstem and spinal cord of all mice with the late onset disease (16–17).

In this report, we used the method of *in situ* hybridization to localize virus-specific RNA in the CNS at early times after intranasal infection. At a few days p.i., MHV-JHM RNA was detected in the trigeminal nerve and some of its associated brainstem nuclei or tracts and in the olfactory system in all mice, whether nursed by immunized or unimmunized dams.

Suckling C57BL/6 mice inoculated intranasally with 6×10^4 PFU MHV-JHM and nursed by unimmunized dams develop encephalitic symptoms at approximately 4 days p.i. and die by 5 days p.i. Few histological changes are present at 3 days p.i., but over the next 2 days, extensive perivascular, parenchymal, and leptomeningeal inflammatory cell infiltrates and widespread areas of necrosis become apparent (*16*).

The brains from acutely infected mice were removed at 3, 4, and 5 days p.i. and analyzed for the presence of MHV-JHM RNA by *in situ* hybridization (*17, 18*) using a ^{35}S-labeled antisense RNA probe synthesized as described previously (*17, 19*). This probe was complementary to all of MHV genes 5 and 6 and portions of genes 4 and 7 (*17, 20*). No annealing occurs with brains or spinal cords from uninfected mice using this method of *in situ* hybridization (*17*). At 3 days p.i., MHV-JHM RNA was invariably present in the olfactory bulb (Fig. 1B). After an additional 24 hr, virus-specific RNA could also be detected in more distal portions of the olfactory

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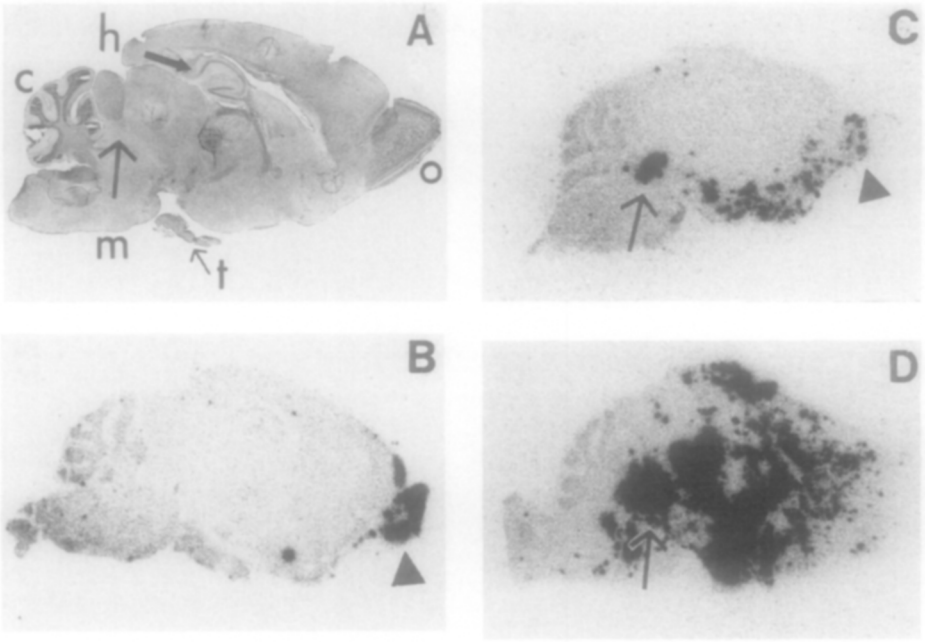

FIG. 1. Temporal appearance of MHV-JHM RNA in mice with acute encephalomyelitis. The offspring of unimmunized C57BL/6 dams (Jackson Laboratories) were inoculated intranasally with 6×10^4 MHV-JHM (obtained from Dr. S. Weiss and grown and titered as previously described (*16*)). Brains were prepared and analyzed by *in situ* hybridization as described previously (*17, 18*). (A) Sagittal section from uninfected mouse stained with hematoxylin and eosin, with landmarks noted. c, cerebellum; o, olfactory bulb; t, trigeminal nerve; m, mesencephalic nucleus of the trigeminal nerve; h, hippocampus. (B–D) Autoradiographs of representative sagittal sections from mice with acute encephalomyelitis at 3 (B), 4 (C), and 5 (D) days p.i. Arrowheads in (B) and (C): olfactory bulb; arrow in (C) and (D): mesencephalic nucleus of the trigeminal nerve.

system including the anterior olfactory nucleus, the olfactory tubercle, the pyriform cortex, the diagonal band of Broca, and the amygdala.

In addition, a prominent site of labeling was present in the brainstem at a position corresponding to the mesencephalic nucleus of the trigeminal nerve in all seven mice which were analyzed (Fig. 1C). By 5 days p.i., the mice were nearly dead and, in agreement with previous results (*4, 16, 21*), MHV-JHM RNA was detected in most parts of the brain, including the hippocampus, hypothalamus, thalamus, and basal ganglia. From these data it appeared that, in mice which developed the acute encephalomyelitis, MHV-JHM entered the CNS via the olfactory and trigeminal tracts, both of which innervate the nose.

Suckling mice inoculated intranasally with the same amount of MHV-JHM and nursed by immunized dams remain asymptomatic although histological examination of their brains at 5–7 days p.i. shows evidence of encephalitis with extensive inflammatory cellular infiltrates. At 3–8 weeks p.i., 40–90% of the mice develop hindlimb paralysis with lymphocytic infiltrates and necrosis particularly prominent in the white matter of the brainstem and spinal cord (*16*). To determine the route of entry of MHV-JHM into the CNS of mice which developed hindlimb paralysis, brains were prepared at early times p.i. from maternal antibody-protected mice and analyzed by *in situ* hybridization.

At 3 days p.i., MHV-JHM RNA was detected only in the olfactory bulb (Fig. 2A) as was also true for mice which developed the acute encephalomyelitis (Fig. 1B). The brains of an additional 9 mice were analyzed from 4 to 7 days p.i. In all of these mice, virus-specific RNA was present in the mesencephalic nucleus and/or the spinal tract of the trigeminal nerve (Fig. 2B). However, in contrast to mice dying from the acute encephalomyelitis, MHV-JHM RNA was not detected in significant quantities in other parts of the brain.

In order to determine if other less virulent strains of MHV also entered the CNS via the olfactory and trigeminal nerves, similar experiments were performed with suckling C57BL/6 mice infected with the less virulent A59 strain of MHV. After intranasal inoculation at 10 days of age with 4×10^5 PFU, 39% (38/97) of the mice died with clinical signs of encephalitis, including hunching, ruffled fur, and irritability, apparent by 5 days p.i. The remainder of the infected mice never developed any signs of infection.

Brains were prepared from 12 MHV-A59-infected mice at 3 to 7 days p.i. and analyzed by *in situ* hybridization as above. By 4 days p.i., virus-specific RNA could be detected in the olfactory system and in the mesencephalic nucleus of the trigeminal nerve of all animals (Fig. 2C). In mice that remained asymptomatic, MHV-A59 had mostly disappeared from the olfactory system by 7 days p.i., but could still be detected at several sites

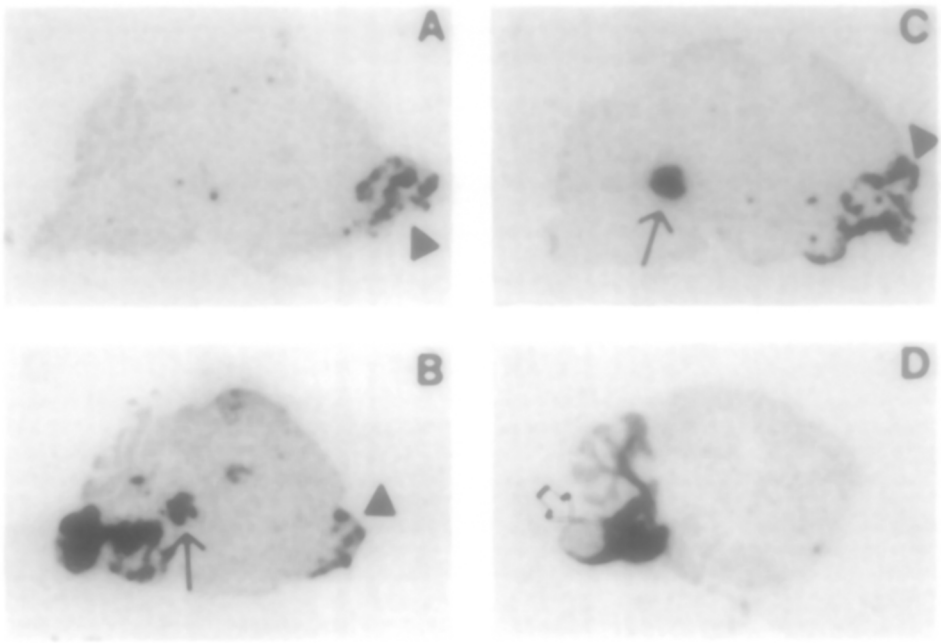

FIG. 2. MHV localization in maternal-antibody protected mice at early times p.i. (A and B) Suckling C57BL/6 mice were infected as above, but were nursed by dams immunized against MHV-JHM as previously described (*16*). Brains from mice at 3 (A) and 7 (B) days p.i. were prepared and analyzed by *in situ* hybridization as above. (C and D) Suckling C57BL/6 were inoculated with 4×10^5 PFU MHV-A59 (obtained from Dr. Susan Weiss) at 10 days of age and were nursed by unimmunized dams. Brains from mice at 4 (C) and 7 (D) days p.i. were prepared and analyzed by *in situ* hybridization as above. The sagittal section shown in (D) is more lateral than the sections shown in (A–C). Arrowhead in (A)–(C): olfactory bulb; arrow in (B) and (C): mesencephalic nucleus of the trigeminal nerve; open arrow in (D): spinal tract of the trigeminal nerve.

in the brainstem including the spinal tract of the trigeminal nerve (Fig. 2D).

These results suggested that after intranasal inoculation of mice with either strain of MHV, virus entered the mouse brain via both the olfactory and the trigeminal nerves. To determine if viral replication could be detected in the trigeminal nerve itself, sections of this nerve were prepared from mice infected with either MHV-JHM or MHV-A59 at 3 to 7 days p.i.

Virus-specific RNA could be detected in 7/13 of the trigeminal nerves which were analyzed. In Fig. 3A, a section of the brain and trigeminal nerve from an asymptomatic MHV-JHM-infected mouse is shown. Viral RNA was readily detected in the trigeminal nerve and in its mesencephalic nucleus. As shown in the figure, viral RNA could also be detected in the limbic cortex of this mouse; the limbic cortex has fiber connections with the olfactory pathway, the other site of viral entry in the mouse. Virus-specific RNA could also be detected in trigeminal nerve isolated from mice infected with MHV-A59 (Fig. 3B). From these experiments it was not possible to determine if MHV replicates in neurons or glia in the trigeminal system.

MHV-JHM can be detected in the brainstem and spinal cord of all mice which develop hindlimb paralysis (*17*) (Fig. 4A). As a first approach to determine the relationship between viral entry, persistence, and amplification resulting in clinical disease, we have analyzed brains from mice with very early signs of hindlimb paralysis by *in situ* hybridization. Our expectation was that if virus persisted at specific sites, it would most readily be detected after some amplification occurred but before spread had occurred to many parts of the brain.

Thus far, we have not been able to detect consistently any specific sites of viral persistence by this approach. However, in some mice with early signs of hindlimb paralysis, MHV-JHM RNA was present primarily in areas of the brain connected to the trigeminal and olfactory nerves, the initial entry sites of virus into the CNS. In the example shown in Figs. 4B and 4C, MHV-JHM RNA was confined to the mesencephalic nucleus of the trigeminal nerve, and to the mamillary nuclei, the alveus of the hippocampus, and the interpeduncular nucleus; the latter three sites are components of the limbic system which in turn is connected with the olfactory pathway.

The data suggest that after intranasal inoculation, MHV enters the mouse CNS via the trigeminal and olfactory nerves. MHV entry appears to be more rapid via the olfactory pathway, since virus-specific RNA is present earlier in the olfactory bulb than in the brainstem nuclei of the trigeminal nerve (Figs. 1B, 2A). Within the trigeminal system, MHV RNA is detected in sensory nuclei and tracts, suggesting that virus spreads initially along sensory rather than motor pathways. Virus presumably enters the CNS via the olfac-

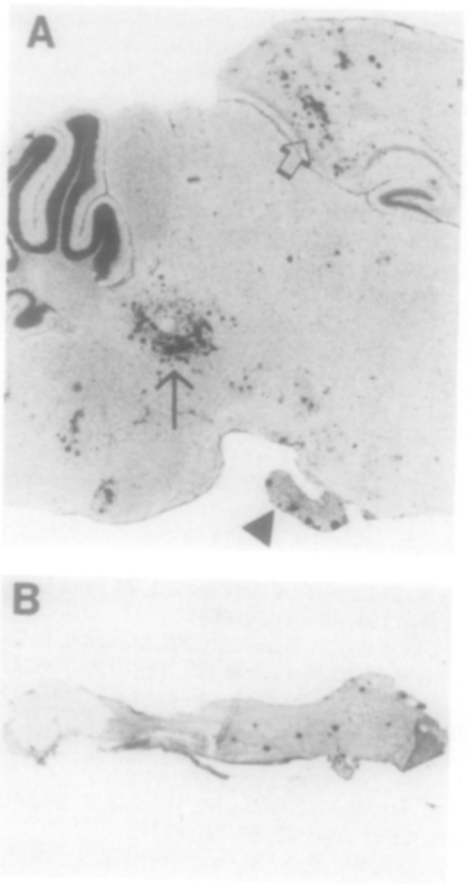

FIG. 3. Viral replication in the trigeminal nerve. (A) Brain and trigeminal nerve were prepared from an asymptomatic MHV-JHM-infected mouse nursed by an immunized dam at 5 days p.i. After analysis by *in situ* hybridization, slides were dipped in Kodak NTB-3 nuclear emulsion prior to exposure for 5 days at 4°. Slides were stained with hematoxylin prior to examination by light microscopy. In this photomicrograph, silver grains corresponding to the location of MHV-JHM RNA are readily visible in the trigeminal nerve (arrowhead), the mesencephalic nucleus of the trigeminal nerve (black arrow) and the limbic cortex (open arrow). Magnification 9.1×. (B) Isolated trigeminal nerve was prepared at 6 days p.i. from an asymptomatic MHV-A59-infected mouse and analyzed as in (A). Clusters of grains are particularly evident on the caudal end of the nerve (right side). Magnification 12.4×.

tory and trigeminal nerves because they innervate the peripheral site of infection. In addition, MHV-JHM appears to enter the CNS via hematogenous spread in the absence of maternal antibody, as evidenced by the diffuse parameningeal infiltrate observed in mice dying from the acute encephalomyelitis (*4, 16, 21*). Such a meningeal cellular infiltrate is not apparent in mice nursed by immunized dams, either at early times p.i. or after development of hindlimb paralysis, consistent with the lack of hematogenous spread under these conditions.

In previous studies of MHV spread in weanling or adult mice after intranasal inoculation, several strains, including MHV-JHM and MHV-A59, have been shown to cause a nasoencephalopathy (*22–24*). The role of the trigeminal nerve as a portal of entry was not discussed, however, in these reports.

Since the presence of maternal antibody does not affect the initial spread of virus to the CNS, this suggests that MHV spreads interneuronally at this stage in agreement with the conclusions of Lavi *et al.* (*10*). Lavi *et al.*, using immunohistochemical methods to detect viral antigen, showed that in weanling mice infected intranasally with MHV-A59, virus appeared to spread via interneuronal transport from the olfactory pathway into the limbic system.

The presence of maternal antibody does prevent the spread of virus to central portions of the brain that occurs in mice which develop acute encephalomyelitis. Since virus is accessible to antibody, this suggests that

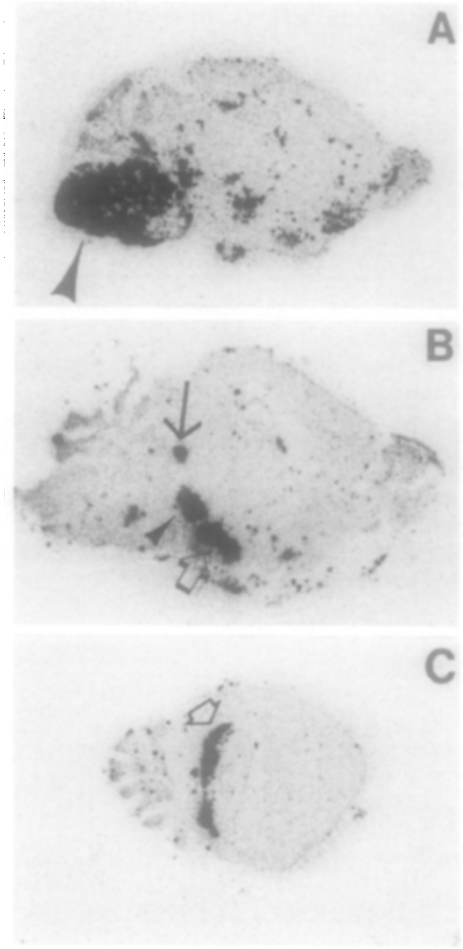

FIG. 4. MHV RNA localization in mice with early signs of hindlimb paralysis. (A) Brain was prepared from a mouse with hindlimb paralysis (28 days p.i.) and analyzed as above. Arrowhead:brainstem. (B and C) Brain was prepared from a mouse with very minimal signs of hindlimb paralysis (18 days p.i.) and analyzed as above. (B) MHV-JHM RNA is present in the mesencephalic nucleus of the trigeminal nerve (black arrow), the mamillary nuclei (clear arrow) and the interpeduncular nucleus (small black arrowhead). (C) Lateral section showing MHV RNA in the alveus of the hippocampus (short, clear arrow).

the widespread dissemination of virus at this stage occurs via extracellular fluid and not interneuronally. However, antibody does not prevent the establishment of viral persistence in asymptomatic mice (*16*).

In most cases of viral encephalitis in humans, virus enters the CNS from the bloodstream (*25*) although both rabies virus and herpes simplex virus (HSV) enter the CNS via interneuronal spread from the periphery (*26, 27*). In mice infected intranasally with HSV, viral antigen was detected in the olfactory system and in the brainstem nuclei of the trigeminal nerve (*28, 29*), in the same distribution we observed after MHV infection. Although no chronic coronavirus infections of the CNS have been identified in humans, the ability of these viruses to spread along neuronal pathways in other mammalian species suggests the possibility that coronaviruses may spread by similar routes in human infections.

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