# Identification of the Spinal Cord as a Major Site of Persistence during Chronic Infection with a Murine Coronavirus

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After intranasal inoculation, mouse hepatitis virus (MHV) gains entry into the central nervous system (CNS) via the olfactory and trigeminal nerves. Under the appropriate conditions, some mice develop clinically apparent demyelinating encephalomyelitis several weeks later, with virus always present in the spinal cord. To determine the pathway by which virus reaches the cord, brains and spinal cords of infected, asymptomatic mice were analyzed by *in situ* hybridization. Viral RNA was always detected in the anterior part of the upper spinal cord. A similar analysis of mice with the recent onset of hindlimb weakness showed that viral RNA was detected in the same location. The results suggest that MHV is transported to the spinal cord via well-defined neuroanatomic pathways and that viral amplification with resultant clinical disease occurs from this site of persistence in the anterior spinal cord. This process of viral amplification may involve the generation of viral variants as has been described for MHV-infected rats. No major changes in viral RNA or protein could be detected when MHV isolated from mice with hindlimb paralysis was analyzed. The data suggest that the generation of viral variants is not important in the pathogenesis of the late onset neurological disease induced by MHV in mice. © 1990 Academic Press, Inc.

#### INTRODUCTION

Mouse hepatitis virus (MHV), a single-stranded positive polarity RNA virus, causes many diseases in susceptible rodents, including gastroenteritis, hepatitis, and a variety of neurological diseases (Siddell et al., 1983). The JHM strain of MHV (MHV-JHM) is neurotropic and causes acute encephalitis and acute and subacute demyelinating diseases in mice and rats (Cheever et al., 1949; Weiner, 1973; Lampert et al., 1973; Nagashima et al., 1978; Sorenson et al., 1980; Stohlman and Weiner, 1981). Suckling mice inoculated intranasally or intracerebrally develop an invariably fatal acute encephalitis; this fatal outcome can be prevented if mice are inoculated with attenuated virus, if they receive an infusion of protective antibody, or if they are nursed by immunized dams (Buchmeier et al., 1984; Pickel et al., 1985; Dalziel et al., 1986; Fleming et al., 1986, 1989; Perlman et al., 1987).

Although suckling C57BL/6 mice inoculated intranasally and nursed by immunized dams are protected from the acute fatal disease, some of them (40–90%) later develop a demyelinating encephalomyelitis characterized clinically by hindlimb paralysis. Previously, we have described the spread of MHV-JHM through the central nervous system (CNS) of suckling mice nursed by unimmunized and immunized dams (Perlman *et al.*, 1988, 1989). After intranasal inoculation, virus enters the brain via the olfactory and trigeminal nerves and spreads along known neuroanatomic pathways. In mice dying from the acute encephalitis, virus is localized in many parts of the brain whereas it remains confined to the sites of entry and their associated structures in asymptomatic mice protected by maternal antibody. The exact localization of MHV-JHM in asymptomatic mice at later times p.i. was not determined in these experiments. In mice which subsequently develop hindlimb paralysis, virus is always present in the spinal cord and less consistently can be detected in specific locations in the brain which are neuroanatomic connections of the initial sites of viral entry. Thus virus can be detected in distant connections of the olfactory nerve such as the hippocampus and mammillary nuclei and in those of the trigeminal nerve such as the caudal thalamus and the cerebral cortex (Perlman et al., 1988, 1989). Virus can easily be isolated from the brains and spi-

virus can easily be isolated from the brains and spinal cords of mice with hindlimb paralysis (Perlman *et al.*, 1987). Characterization of these mouse isolates has not been published, but MHV-JHM isolated from rats with a similar neurological disease (Nagashima *et al.*, 1978; Sorensen *et al.*, 1980) causes an attenuated disease on subsequent inoculation into susceptible rats and contains a shortened spike (S) glycoprotein and mRNA (Morris *et al.*, 1989). In other studies, a longer glycoprotein has been associated with increased neurovirulence in young rats (Taguchi *et al.*, 1985).

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In this report, we describe the location of viral RNA in the CNS of asymptomatic mice at later times p.i. and compare this to the distribution of viral RNA in mice with the early onset of hindlimb paralysis. We also show that the virus isolated from the brains and spinal cords of mice with hindlimb paralysis appears identical to the initial infecting strain of virus by clinical and biochemical criteria.

### MATERIALS AND METHODS

#### Animals and virus

C57BL/6 mice, used in all studies, were negative for MHV antibody prior to experimental manipulation. Mice were immunized as described previously (Perlman *et al.*, 1987). MHV-JHM, originally obtained from Dr. S. Weiss, was plaque-purified and grown as described previously (Perlman *et al.*, 1987). To obtain isolates from infected animals, brains and spinal cords were removed from individual mice with hindlimb paralysis, sonicated in Dulbecco's minimum essential media (DMEM) without serum, and clarified by centrifugation. The supernatant was passaged onto BALB/c 17CL-1 cells; virus from the second or third passage was used in all studies. Virus was titered on L-2 cells as described previously (Perlman *et al.*, 1987).

#### In situ hybridization

*In situ* hybridization was performed using an <sup>35</sup>S-labeled antisense RNA probe as previously described (Perlman *et al.*, 1988). The RNA probe was complementary to genes 5 and 6, and to 200 bases of genes 4 and 7. Slides were initially exposed to X-ray film (Kodak XAR) for 1–3 days at 4° and were then dipped in NTB-2 nuclear emulsion prior to exposure for 1–2 weeks at 4°. Slides were stained with hematoxylin prior to examination by light microscopy. For each experiment, a negative (uninfected brain) and a positive (mouse dying from acute encephalitis) control were analyzed in parallel. No annealing was detected by film autoradiography when uninfected brains were annealed with the MHV probe.

#### **RNA blot analysis**

RNA was isolated from infected brains, spinal cords, and tissue culture cells using the guanidinium isothiocyanate–cesium chloride method (Maniatis *et al.*, 1982). RNA was fractionated by agarose gel electrophoresis, transferred to nitrocellulose or nylon membrane filters (Nytran, Schleicher & Schuell), and probed with <sup>32</sup>P-labeled antisense RNA. The same antisense RNA probe was used as in the *in situ* hybridization experiments.

# ELISA

Different viral isolates were compared using a solidphase ELISA with a panel of monoclonal antibodies directed against the S glycoprotein of MHV-JHM, kindly provided by Dr. Michael Buchmeier. Microtiter plates (Nunc) were coated with 0.1 ml/well of partially purified MHV-JHM. For the preparation of MHV-JHM, infected 17CL-1 cells were freeze-thawed, heavy particulate material was removed by centrifugation, and virus prepared by centrifugation at 100,000 g in the Beckman SW56.1 rotor. Virus was resuspended in DMEM without serum and adsorbed overnight at room temperature to the plates. Plates were then blocked with 5% skimmed milk for 1 hr at room temperature, washed with phosphate-buffered saline (pH 7.2) with 0.05% Tween 20 (PBS-T), and incubated with dilutions of the monoclonal antibodies. After washing with PBS-T, the plates were incubated successively with 0.1 ml/well biotin-conjugated goat anti-mouse immunoglobulin antibody (1:1000 dilution, Zymed), with streptavidin-alkaline phosphatase conjugate (1:2000, Zymed), and with p-nitrophenyl phosphate disodium (Sigma). The reaction was stopped with 0.3 N NaOH and the plates read with an ELISA reader (Bio-Rad Model 2550).

#### RESULTS

#### Location of virus in asymptomatic mice

At early times after intranasal inoculation, viral RNA is readily detected in the trigeminal nerve, olfactory bulb, and their connections in all mice (Perlman *et al.*, 1989). In mice protected from the acute encephalitis by maternal antibody, virus spreads to the neuroanatomic connections of these sites of entry, including the spinal tract and nucleus of the trigeminal nerve, the hippocampus, and the limbic cortex. Forty to ninety percent of the mice later develop hindlimb paralysis, and, in these animals, viral RNA can always be detected in the spinal cord, and less consistently in certain locations in the brain (Perlman *et al.*, 1988, 1989).

To determine the relationship between the subsequent progression of virus in asymptomatic mice and the location of virus in mice with the late onset neurological disease, sagittal sections of brains and spinal cords from seven asymptomatic mice at 15–16 days p.i. were analyzed by *in situ* hybridization as previously described (Perlman *et al.*, 1988). The concentration of viral RNA in the brains and spinal cords from asymptomatic mice was no more than 1% (mean of 0.4% which is equivalent to approximately 10 pg/µg spinal cord RNA) of that present in the spinal cords of mice with hindlimb paralysis when analyzed by blot analysis (data not shown). However, viral RNA could readily be



Fig. 1. Location of MHV-JHM RNA in asymptomatic mice. Maternal antibody-protected mice were inoculated at 10 days of age with MHV-JHM. Brains and spinal cords were removed and analyzed by *in situ* hybridization as described previously (Perlman *et al.*, 1988). (A) Frozen sagittal section stained with hematoxylin and eosin to show orientation of brain and spinal cord. Ce, cerebellum; Sc, spinal cord; O, olfactory lobe. (B) Autoradiograph of mouse analyzed 15 days after intranasal inoculation. (C) Analysis 12 days after inoculation into the hindlimb. (D) Analysis 10 days after intraperitoneal inoculation. Arrows indicate labeling in anterior portion of upper spinal cord.

detected in the anterior part of the spinal cord (Fig. 1B) in all asymptomatic mice studied. Microscopic examination of these sections revealed small amounts of viral RNA in other parts of the CNS, but the highest concentration was always found in the upper spinal cord at this stage of the infection.

To determine if this tropism for the spinal cord occurs after other routes of inoculation, mice were inoculated with MHV-JHM in the hindlimb and intraperitoneally (i.p.). Suckling mice inoculated in the hindlimb with  $1.5-3 \times 10^5$  PFU MHV-JHM did not develop either acute encephalitis or hindlimb paralysis. When the brains and spinal cords of these mice were analyzed at 10-16 days p.i. by *in situ* hybridization, viral RNA could be detected in the anterior part of the spinal cord in 3/7 mice (Fig. 1C). It is conceivable that, in this case, virus reached the spinal cord from peripheral nerves via a transneuronal route, although we could not detect virus at earlier times p.i. in the lower segments of the spinal cord at sites corresponding to the sites of entry of the hindlimb nerves (data not shown).

It has been shown that suckling mice develop acute encephalitis after intraperitoneal inoculation (Cheever *et al.*, 1949; Bailey *et al.*, 1949). However, suckling mice inoculated i.p. at 10 days of age with  $5-10 \times 10^4$  PFU of MHV-JHM and nursed by immunized dams were partially protected against the acute encephalitis. We observed that 33% (16/49) developed acute encephalitis, 51% (25/49) remained asymptomatic, and 16% (8/49) developed hindlimb paralysis. When i.p.-inoculated asymptomatic mice were analyzed by *in situ* hybridization at 10–15 days p.i., viral RNA was readily detected in the anterior part of the upper spinal cord in 3/4 mice (Fig. 1D).

To localize MHV-JHM RNA more precisely in the spinal cord, coronal sections from asymptomatic mice inoculated intranasally were prepared and analyzed by *in situ* hybridization. As shown in Figs. 2 and 3, viral RNA was most evident in the ventrolateral part of the cord, corresponding to the white matter around the ventral (motor) horn of the spinal cord. Microscopic analysis of other sections showed that viral RNA could also be detected in smaller quantities in the dorsolateral and dorsal (sensory) zones of the spinal cord. Thus, MHV-JHM RNA was present dorsolaterally in the substantia gelatinosa (Fig. 3C) and in the dorsal (posterior) white



Fig. 2. Location of virus in coronal sections of spinal cords. Coronal sections were prepared from a maternal antibody-protected mouse at 15 days after intranasal inoculation and analyzed by *in situ* hybridization. (A) Frozen section stained with hematoxylin and eosin to show orientation. Sc, spinal cord; Vent, ventral; Dors, dorsal. (B) Autoradiograph of coronal section, in which MHV-JHM is localized to the ventral and lateral parts of the spinal cord.

column (Fig. 3D). The substantia gelatinosa in the upper cord is continuous with the nucleus of the spinal tract of the trigeminal nerve.

# Relationship between location of viral RNA in asymptomatic and symptomatic mice

In mice which develop hindlimb paralysis, MHV-JHM RNA is always present in the spinal cord, and variably present in specific parts of the brain (Perlman et al., 1988). To determine the earliest sites of viral replication in mice which develop the late onset neurological disease, brains and spinal cords from mice with very early hindlimb weakness (clinically apparent for less than 24 h) were analyzed by in situ hybridization. MHV-JHM RNA could not be detected reproducibly at any single site within the brains of these mice as reported previously (Perlman et al., 1989). However, when spinal cords were analyzed, viral RNA could be detected in the anterior part of the upper cord in all four mice examined (Fig. 4). Thus viral RNA was localized to the same site in asymptomatic mice and in mice with the onset of hindlimb paresis.

# Characterization of MHV-JHM isolated from infected mice

In the next set of experiments we determined if virus isolated from mice with hindlimb paralysis became less neurovirulent during the course of infection, as has been described in rats infected with MHV-JHM (Morris *et al.*, 1989). For this purpose, virus isolated from C57BL/6 mice with hindlimb paralysis was compared to the input virus by several criteria.

*I. Clinical.* MHV-JHM was prepared from infected brains and spinal cords of mice with hindlimb paralysis and passaged in tissue culture cells. Eight suckling C57BL/6 mice were inoculated intranasally with 6  $\times$  10<sup>4</sup> PFU of a brain isolate and five were inoculated with the same amount of a spinal cord isolate. These mice, nursed by unimmunized dams, all succumbed to acute encephalitis by 6 days p.i.; the temporal appearance of clinical disease could not be distinguished from littermates inoculated with the input strain of virus. Thus an attenuated strain of MHV-JHM was not selected by passage through mice.

*II. RNA analysis.* The decrease in neurovirulence observed in rats was associated with a smaller RNA coding for the spike glycoprotein (mRNA 3). To determine



FIG. 3. Microscopic localization of MHV-JHM in coronal sections of spinal cord. Coronal sections from a maternal antibody-protected mouse at 15 days after intranasal inoculation were analyzed by *in situ* hybridization and emulsion autoradiography. (A) Section stained with hematoxylin and eosin to show orientation of spinal cord. Vent, ventral. B, C, and D refer to other panels of figure. (B) Ventral spinal cord showing several areas of MHV-JHM labeling. (C) Single intense area of staining in substantia gelatinosa. (D) Several areas of MHV-JHM labeling in dorsal column. Magnification bar, 100 µm.

if such a change occurred during passage in the C57BL/6 mice, virus was harvested from the brains and spinal cords of mice with hindlimb paralysis as described under Materials and Methods. RNA was prepared from tissue culture cells infected with isolates from different mice and analyzed by formaldehydeagarose gel electrophoresis. As shown in Fig. 5A, there was no obvious difference in mobility between RNA 3 from the input virus and RNA 3 from the animal isolates. Six animal isolates (three from the brain and three from the spinal cord) were analyzed in these experiments. As control, RNA isolated from cells infected with the A59 strain of MHV was included in these analyses. As shown in the figure, MHV-A59 RNA 3 has a slightly faster mobility than MHV-JHM or any of the mouse isolates, consistent with the previously reported deletion of approximately 155 nucleotides (Parker et al., 1989).

In the above experiments, virus was passaged through tissue culture cells before analysis. To eliminate the possibility that passage through these cells selected against the growth of a variant strain, we analyzed RNA directly isolated from infected brains and spinal cords. As shown in Fig. 5B, no difference existed in mobility between RNA 3 isolated directly from mice and that isolated from tissue culture cells infected with the initial strain of MHV-JHM.

*III. Protein analysis.* The S glycoprotein isolated from some of the less neurovirulent strains of MHV-JHM is both shortened and lacking specific epitopes as compared to the more virulent strains (Taguchi *et al.*, 1985; Morris *et al.*, 1989, Taguchi and Fleming, 1989). Using a panel of monoclonal antibodies directed against the S glycoprotein, we compared the virus isolated from mice with hindlimb paralysis to the initial strain of MHV-JHM. As shown in Fig. 6, all of the monoclonal antibodies recognized all of the isolates of MHV-JHM. In particular, monoclonal antibody 4B11.6, which appears to recognize an epitope (Epitope E2C) associated with neurovirulence (Dalziel *et al.*, 1986), reacted with all of the strains of virus, but did not react with MHV-A59, in agreement with previous results (Talbot and Buchmeier, 1985).

Thus, by all criteria tested, no differences could be detected between the initial strain of virus and MHV-



Fig. 4. Comparison of labeling in asymptomatic mice and in mice with recent onset of hindlimb paresis. Brains and spinal cords were prepared from asymptomatic mice at 15 days (B) and from a mouse with recent onset of hindlimb paresis at 24 days after intranasal inoculation (C). Sagittal sections were analyzed by *in situ* hybridization. (A) Section stained with hematoxylin and eosin for purpose of orientation. (B) and (C) Darkfield micrographs of boxed area of spinal cord shown in (A). Labeling is apparent in anterior part of spinal cord in both cases. Magnification bar, 1 mm.

JHM isolated from the brains and spinal cords of mice with hindlimb paralysis.

# DISCUSSION

Our results show that MHV-JHM spreads within the brain and spinal cord of infected mice via specific path-



Fig. 5. Gel analysis of MHV-JHM RNA from mouse CNS isolates. (A) Brains and spinal cords were removed from mice with hindlimb paralysis and virus was prepared as described under Materials and Methods. L-2 cells were infected with the different isolates of MHV (m.o.i. 0.1 to 0.3). Viral RNA was prepared at 11 hr p.i. and analyzed as described under Materials and Methods. B2, B3 are two brain isolates whereas SC1 and SC2 are spinal cord isolates. WT refers to the strain of virus used to infect the mice. A59 refers to MHV-A59. Viral RNAs, labeled 1–7, are indicated as well; in specific, RNA 3 codes for the S glycoprotein. (B) A brain and spinal cord from a mouse with hindlimb paralysis was removed and immediately homogenized in guanidinium isothiocyanate as above. RNA was then analyzed as in (A). Br, brain RNA; Sc, spinal cord RNA; WT, RNA from tissue culture cells infected with the initial strain of virus.

ways. Following intranasal inoculation, the virus reaches the brain via the trigeminal and olfactory nerves, and rapidly spreads to neuroanatomic struc-



Fig. 6. ELISA comparing initial strain of virus and mouse isolates. Virus isolated from mice and passaged through tissue culture cells was analyzed by ELISA as described under Materials and Methods. Specific antibodies are labeled as described previously (Buchmeier *et al.*, 1984). B1, B2, brain isolates; SC1, SC2, SC3, spinal cord isolates. Some of the same isolates were used in Fig. 5A and in this figure. WT, Initial strain of MHV-JHM used to infect the mice; A59, attenuated MHV-A59 strain of virus. Solid boxes represent greater than 85% binding as compared to wild type; open boxes represent less than 10% binding.



Fig. 7. Schematic drawing of sites of localization and routes of spread of virus through the mouse CNS. (A) MHV-JHM enters the CNS via the first division ( $V_1$ ) of the trigeminal nerve to its sensory nuclei (shaded area). Continuity of the nucleus of the spinal tract of the trigeminal nerve with the dorsal horn (substantia gelatinosa) of the upper spinal cord is shown.  $C_1$ ,  $C_2$ , first and second cervical spinal segments;  $V_1$ ,  $V_2$ ,  $V_3$ , first, second, and third divisions of trigeminal nerve. (B) Virus also may pass from the trigeminal system to reticular nuclei in the pons and the medulla and from there enter the spinal cord via the reticulospinal tract. Reticulospinal fibers terminate on cells in the anterior portion of the spinal cord.

tures related to these nerves. This occurs even in the presence of maternal antibody, which limits extracellular spread (Perlman *et al.*, 1989). The same progression through the olfactory system has been described in mice infected with MHV-A59 (Lavi *et al.*, 1988).

The virus is also detected in the spinal cord of asymptomatic, infected mice. In the spinal cord, virus is localized largely in the ventral (motor, anterior) part and to a lesser extent in the dorsolateral or dorsal (sensory) parts. A likely explanation for the ventral cord localization of virus is that the virus is transported from the trigeminal nerve to the sensory trigeminal nuclei in the brainstem (Fig. 7A) and from there to the reticular nuclei of the pons and medulla oblongata (Barr and Kiernan, 1988) which are known to project via the reticulospinal tract (Fig. 7B) to the spinal motor neurons in the ventral horn of the spinal cord. The localization of virus in the white matter of the anterior cord corresponds to the anatomic localization of the reticulospinal tract in the spinal cord. An alternative, though less likely route, is via the olfactory-reticular connections. Olfactory stimulation has been shown by physiologic methods to evoke activity in the reticular formation possibly mediated by corticoreticular fibers arising from olfactory areas of the cerebral cortex (Barr and Kiernan, 1988).

The dorsolateral localization of virus in the spinal cord in all probability reflects virus presence in the pars caudalis of the nucleus of the spinal tract of the trigeminal nerve, which is continuous with the substantia gelatinosa located in the upper cord (Figs. 3 and 7A).

Alternative explanations for the spread of virus to specific parts of the spinal cord also exist. MHV-JHM may spread throughout the CNS via the cerebrospinal fluid (CSF), with replication only at those sites containing specific receptors for MHV-JHM. This model predicts that free virus should be present in the CSF at some time during the course of MHV-JHM infection. Alternatively, since MHV-JHM has been shown to infect rat spinal ganglionic neurons (Sorensen and Dales, 1985), virus could conceivably spread to specific areas of the spinal cord after initial amplification in spinal ganglia. Infection of spinal ganglia may be particularly important in the pathogenesis of CNS infection following intraperitoneal inoculation. Experiments in which specific parts of the trigeminal and olfactory pathways are transected prior to intranasal inoculation should help distinguish between models involving neuroanatomic spread and those involving spread via the CSF or spinal ganglia.

Once MHV-JHM has spread to specific sites in the CNS, the development of the late onset neurological disease is dependent upon increased viral replication which occurs primarily at these locations. Thus, viral RNA can be detected in the upper part of the spinal cord in asymptomatic mice; viral replication has increased greatly in mice with early signs of hindlimb paresis, but viral RNA is still localized to the same part of the spinal cord. At later times after the development of hindlimb paralysis, virus can be detected throughout the spinal cord (Perlman *et al.*, 1988).

The pathogenesis of the late onset neurological disease involves direct lysis of oligodendrocytes with minimal neuronal involvement (Lampert *et al.*, 1973; Knobler *et al.*, 1981a). Consistent with this, myelin gene transcripts are reduced within demyelinating lesions caused by the closely related A59 strain of MHV (Jordan *et al.*, 1989). This is probably due to direct destruction of oligodendrocytes by MHV. Infection of neurons appears to be important primarily in the invariably fatal, acute encephalitis seen at early times p.i. (Knobler *et al.*, 1981a,b).

The tropism which MHV-JHM shows for the spinal cord is in contrast to the results obtained after infection of animals or humans with herpes simplex virus (HSV). After intranasal inoculation in the rabbit, HSV undergoes latency in the trigeminal ganglion and olfactory neurons and only reactivates after immunosuppression with cyclophosphamide and dexamethasone (Stroop and Schaefer, 1986). After reactivation, virus migrates to the temporal lobe, resulting in a severe encephalitis. A similar process may occur in humans who develop HSV encephalitis (Corey and Spear, 1986; Stroop, 1986). Thus it appears that HSV and MHV establish latency or persistence in the same locations, but that HSV preferentially reactivates from the trigeminal or olfactory systems in the brain, whereas MHV is amplified most commonly from a site of persistence in the spinal cord.

The factors important for viral persistence and amplification are not known. In other viral infections, generation of variants during the course of infection may help the virus evade immune surveillance and thus maintain viral persistence (Oldstone, 1989). The pathogenesis of MHV persistence in rats may include such changes, since virus with a deletion in the S glycoprotein and its corresponding mRNA has been shown to correlate with decreased ability to cause acute encephalitis (Taguchi et al., 1985; Morris et al., 1989). As documented above, we could find no evidence that such large deletions or such changes in pathogenicity occurred in MHV-JHM during the course of persistent infection in the mouse. It should be noted that small deletions or single base mutations, which could have biological importance, might not have been detected in these experiments.

An inability of the mouse immune system to eradicate the infection is most likely important in the pathogenesis of MHV persistence. High levels of antibody do not protect either mice or rats from MHV-JHM-induced demyelination (Stohlman and Weiner, 1981; Sorensen *et al.*, 1984; Dorries *et al.*, 1987). Although cell-mediated immunity is believed to be most important in eradicating an MHV infection (Sussman *et al.*, 1989; Zimmer and Dales, 1989), little is known about either the cellmediated immune response to MHV or the status of antigen presentation in persistently infected mice. Such information will be crucial to our understanding of viral persistence and amplification in these animals.

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