

Short Communication

Further observations on coronavirus infection of primate CNS

Ronald S Murray^{1,2}, Guang-Yun Cai⁴, Kenneth F Soike³ and Gary F Cabirac^{4,5}

¹Colorado Multiple Sclerosis Center, Englewood, Colorado, 80110; ²National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80262; ³Tulane Regional Primate Research Center, Covington, Louisiana 70433; ⁴Rocky Mountain MS Center, Englewood, CO 80110; and ⁵Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262, USA

Previously we demonstrated that intracerebral (IC) inoculation of a murine coronavirus, MHV-JHM, into two species of primates can result in acute encephalomyelitis (Murray *et al.*, 1992a). Infectious virus isolated from acutely infected animals, designated JHM-OMP1, was inoculated IC into a second group of monkeys. In this report we describe observations on the acutely infected animals and those surviving the acute infection that were sacrificed at later times post-infection. Results from dual *in situ* hybridization/immunohistochemistry screening of tissues show that astrocytes are target cells in white matter lesions during acute infection. In animals sacrificed 150 days post-infection, areas of demyelinated gliotic lesions, prominent in the spinal cord, were seen throughout the neuraxis. No virus products were detected in these late-infection lesions.

Keywords: brain disease; demyelinating disease; virus disease; astrocytes

Coronavirus infection in rodents is widely used as a model for studying virus induced CNS demyelination. Speculation on the role of coronaviruses and other viruses in human demyelinating diseases such as multiple sclerosis (MS) has existed for many years. However, coronaviruses have never been shown to cause CNS disease in human. In 1980 there was a report of coronavirus isolation from MS patient autopsy brains (Burks *et al.*, 1980) but subsequent analysis of these isolates showed that they were more closely related to the prototypic murine hepatitis viruses (MHV) than to identified human coronaviruses (Gerdes *et al.*, 1981; Weiss, 1983). Therefore it was assumed that these putative human CNS coronaviruses were contaminants originating from the culture systems used for their isolation. More recently, the idea that human CNS tissue is susceptible to infection by 'murine' coronaviruses was re-introduced. Results from our laboratory showing detection of coronavirus products in MS autopsy brain tissue by *in situ* nucleic acid hybridization and monoclonal antibody stain-

ing suggested that murine-like viruses are capable of infecting the human CNS (Murray *et al.*, 1992b). In addition, we reported that MHV strain JHM and one of the putative human CNS coronavirus isolates could replicate, disseminate and produce acute CNS disease in two species of primates following IC inoculation (Murray *et al.*, 1992a).

To expand our knowledge of murine coronavirus pathogenesis in primates we inoculated a primate CNS passaged isolate, designated JHM-OMP1, and performed additional studies on acutely infected CNS tissue and on tissue isolated at later times post-infection. Owl monkeys were chosen for this study because this is the species that was used to obtain the passaged isolate. Isolation, propagation, titring of JHM-OMP1 and preparation of the IC inoculum has previously been described (Murray *et al.*, 1992a); the titer of the inoculum was approximately 10^5 TCID₅₀/ml. Five outbred adult Owl Monkeys (OM) were inoculated IC with 0.5 ml through a burr hole into the right subcortical white matter. Two animals were sham inoculated with a suspension prepared from uninfected DBT cells. The monkeys were observed daily for any clinical signs of infection. Blood was drawn from each animal on 7, 14, 21, 35, 60 and 90 days post-infection (dpi) for infectious virus and neutralizing antibody assays. Two virus inoculated animals, K191 and K063, were sacrificed 10 and 12 dpi, respectively, due to

Correspondence: R Murray

Received 13 February 1996; revised 10 September 1996; accepted 10 October 1996

severe neurological impairment; these were designated acutely infected animals. Two of the remaining three virus inoculated animals, K189 and K171, and one of the sham inoculated control animals were re-inoculated IC with JHM-OMp1 after 90 days. All remaining animals were sacrificed 150 dpi (60 days post second inoculation). At the time of sacrifice brain and spinal cord tissue plus cerebrospinal fluid (CSF) samples were taken for infectious virus assays. A portion of each animal's brain and spinal cord was snap-frozen while the remaining tissue was fixed in formalin and paraffin embedded. RNA was extracted from frozen tissues for later Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Sections cut from formalin-fixed tissues were analyzed by *in situ* hybridization, immunostaining with JHM-specific monoclonal antibody, and hematoxylin and eosin and luxol-fast blue staining. Detailed descriptions of the aforementioned methods have been previously described (Vafai *et al.*, 1988; Murray *et al.*, 1992a,b).

Table 1 shows results of neutralizing antibody and infectious virus assays detection of viral RNA/antigen and summaries of pathology for each animal. The results for animals K191 and K063 have previously been reported and discussed (Murray *et al.*, 1992a) and are presented in this table for comparison to those obtained with the animals sacrificed at 150 dpi (K189, K171 and K072). There were two main differences between the two animals sacrificed during acute disease and those sacrificed at 150 dpi. First, infectious virus could be isolated from the acutely infected animals but not from those sacrificed at later times. Second, the pathology observed was different between the two sets of animals. The pathology observed in the acutely infected animals has been described pre-

viously (Murray *et al.*, 1992a) while that observed for the animals sacrificed at 150 dpi was as follows. All three animals had foci of perivascular demyelination in the neuraxis with prominent areas of multilevel demyelination in the dorsolateral spinal cord. Figure 1a shows an area of decreased luxol-fast blue indicating demyelination in a section of spinal cord obtained from animal K189. These areas of spinal cord demyelination were accompanied by gliosis characterized by reactive astrocytes as shown by staining with a glial fibrillary acidic protein (GFAP)-specific monoclonal antibody (Figure 1b). Immunostaining for macrophages and monocytes was negative in these areas (data not shown). Bielschowsky silver staining (Lowe and Cox, 1990) of these spinal cord lesions showed that axons within the demyelinated areas were intact (Figure 1c and d) indicating that demyelination was not secondary to Wallerian degeneration caused by the cerebral inoculation. It should be noted that there was no difference in pathology between animal K072, the animal that received one IC inoculation, and the two animals, K189 and K171, that received two IC inoculations.

We have previously shown that viral RNA and antigen could be detected in the CNS of acutely infected animals (Murray *et al.*, 1992a). However, in the animals surviving acute infection and sacrificed at later times, K072, K171 and K189, viral RNA could not be detected in areas of demyelination by *in situ* hybridization. In addition, JHM-specific products could not be amplified by RT-PCR using RNA extracted from these tissues. RT-PCR was performed on RNA extracted from frozen tissue as previously described (Murray *et al.*, 1992a). Viral antigen could not be detected by immunostaining of these areas with JHM-specific monoclonal antibody. This lack of recoverable infectious virus

Table 1

Animal ^a	Sacrificed ^b	Ab ^c	Infectious virus assays ^d				RNA/Antigen ^e		MAB	H&E/LFB ^f
			Brain	CSF	Blood	Northern	ISH	PCR		
K191	10	—	+	—	—	+	+	+	+	Severe encephalomyelitis
K063	12	—	+	—	+	+	+	+	+	Severe encephalomyelitis
K189*	150	1:20	—	—	—	ND	—	—	—	Demyelination in brain and spinal cord; mild meningitis
K171*	150	1:10	—	—	—	ND	—	—	—	Demyelination in brain and spinal cord; mild meningitis
K072	150	—	—	—	—	ND	—	—	—	Demyelination in brain and spinal cord; mild meningitis

^aVirus infected animals. Results for sham inoculated animals are not shown

^bDays post-infection. Animals K191 and K063 were sacrificed after neurological dysfunction was observed

^cNeutralizing antibody titers. Values represent the highest titer measured from all time points

^dBrain and CSF samples were assayed at time of sacrifice. Assays on blood samples were done for all time points described in the text

^eDetection of RNA and virus nucleocapsid protein: Northern, Northern analysis; ISH, *In situ* hybridization; PCR, polymerase chain reaction; MAB, Immunostaining with monoclonal antibody J.3.1

^fPathology determined by observation of hematoxylin and eosin and luxol-fast blue stained tissue sections

*These two animals received a second IC inoculation of JHM-OMp1 at 90 days post-infection

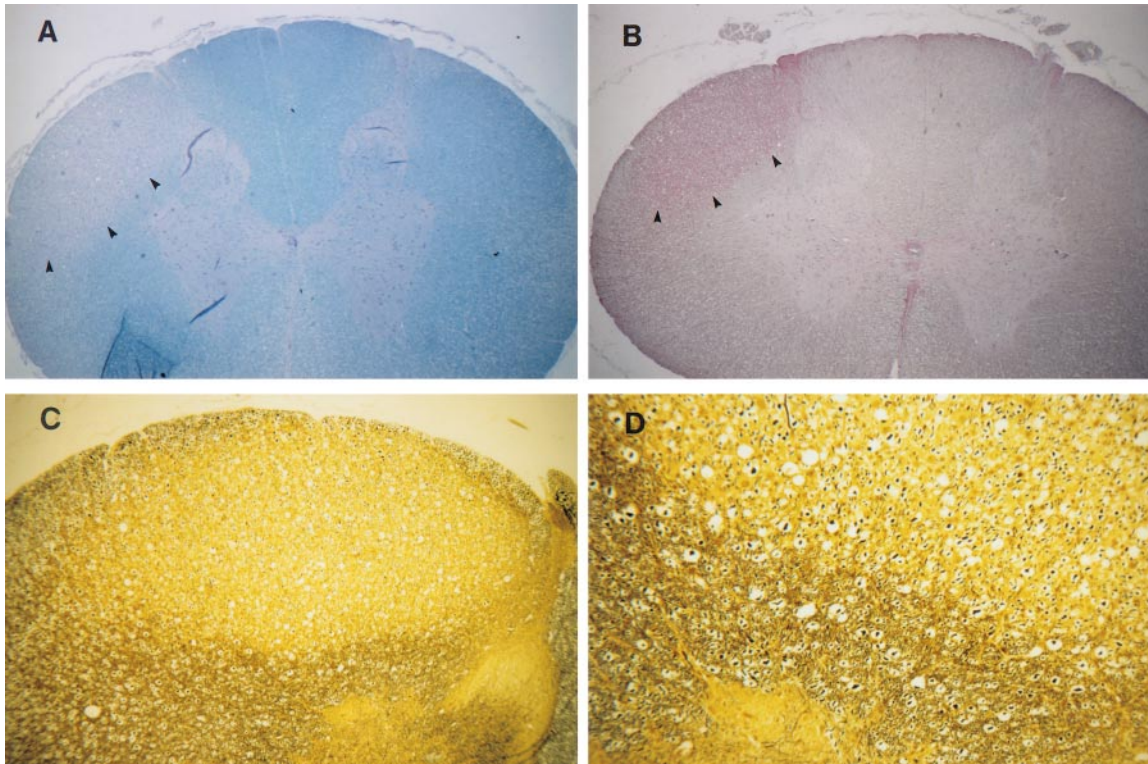


Figure 1 Histopathology, immunostaining and *in situ* hybridization of primate CNS tissues. (a) Decreased Luxol-fast blue staining for myelin in dorsolateral column of spinal cord obtained from animal sacrificed 150 dayspost-infection. Lesion is outlined by arrowheads (Mag.25×). (b) Extensive astrocyte proliferation (darkened region outlined by small arrowheads) as shown by anti-GFAP immunostain. This section was adjacent to that shown in (a) and is lightly counterstained with hematoxylin (Mag.25×). (c) Bielschowsky silver staining of spinal cord showing area of demyelination (Mag.100×). (d) Higher magnification of silver stained tissue shown in (c) demonstrating intact axons in the demyelinated (lighter staining) and un-affected (darker staining) areas (Mag.250×).

during the chronic phase of disease has been observed in mice that were inoculated with MHV strains (Stohlman and Weiner, 1981; Lavi *et al.*, 1984).

In our previous report we described the infection of primate CNS with JHM that was passaged on mouse cells in culture (Murray *et al.*, 1992a). We showed by *in situ* hybridization that in acutely infected animals cerebellar purkinje cells and cortical neurons contained coronavirus RNA. Infected white matter cells containing viral RNA were not specifically identified in this original study. In the current study, tissue from the two acutely infected animals, K191 and K063, was used for further identification of infected white matter cell types. This was accomplished using a dual *in situ* hybridization-immunostaining method. Utilizing this combination of nucleic acid and antigen detection we found that JHM-OMP1 could be detected in astrocytes during the acute white matter infection. A monoclonal antibody specific for GFAP (clone GA5, Boehringer Mannheim) was used for immunostaining of astrocytes. A commercially available kit, Super Sensitive biotin-streptavidin kit (BioGenex), was used according to the manu-

facturers instructions to stain the primary antibody with chromophore. Antibody labeled tissue sections were washed with deionized water, treated with 0.1 M HCl at room temperature for 20 min and then incubated with proteinase K (2 µg/ml in 10 mM Tris-Cl, pH 7.4, 2 mM CaCl₂) at 37°C for 10 min. The tissue sections were then processed for *in situ* hybridization using a radiolabeled MHV-specific cDNA probe. Hybridization was as previously described (Vafai *et al.*, 1988; Murray *et al.*, 1992b). Figure 2 shows positive GFAP staining of astrocytes (red colored) in tissue obtained from an infected animal with co-localization of silver grains over some of these cells thus indicating that viral RNA can be detected in astrocytes. From these results, it appears that oligodendrocytes were not the primary target cell for JHM-OMP1.

These results show that astrocytes are a primary target cell for JHM-OMP1 infection in Owl monkeys during acute infection. This is similar to MHV-infection in the rodent CNS where astrocytes are one of numerous cell types that support virus replication (Perlman and Ries, 1987; Massa *et al.*, 1986). In our studies of acutely infected primates, inflammatory and demyelinating areas containing

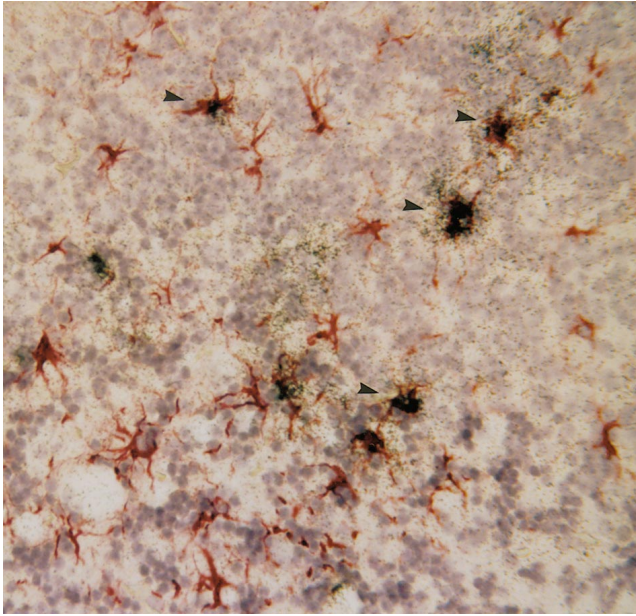


Figure 2 Dual *in situ* hybridization and α -GFAP staining immunostaining showing silver grain development over α -GFAP positive stained cells (red) indicating virus infection of astrocytes (Mag.400 \times). Arrowheads show a number of astrocytes positive for viral RNA.

viral RNA or antigen were always accompanied by reactive astrocytes. Infected astrocytes were not detected in the spinal cords from the two acutely infected animals but it is possible that these two animals were sacrificed before active infection had spread to the spinal cord. Therefore, an assumption can be made that the astrogliosis and demyelination observed in the spinal cord lesions in the animals sacrificed at later times were the result of virus infection of astrocytes or other cell types. Due to the later time of sacrifice we did not observe viral products or an inflammatory infiltrate in late areas of demyelination. Presumably, demyelination is

attributable to direct viral effects and/or autoimmune mechanisms and not Wallerian degeneration as shown in Figure 1d. There is support for both mechanisms operating in rodent systems (Lampert *et al.*, 1973; Weiner, 1973; Wang *et al.*, 1990; Fleming *et al.*, 1993; Watanabe *et al.*, 1983). The adoptive transfer of lymphocytes isolated from JHM infected rats, restimulated with myelin basic protein (MBP), induces in the recipients lesions resembling those of experimental allergic encephalomyelitis (Watanabe *et al.*, 1983). However, these T cells are not simultaneously reactive against coronavirus and MBP thus ruling out 'molecular mimicry' as a unifying mechanism. This study does strongly imply that a concomitant immune mediated demyelination is precipitated by a coronavirus infection and suggests that a similar mechanism could be operating in human demyelinating diseases. Recently, it was shown that MHV infection of murine astrocytes *in vivo* leads to the production of inflammatory cytokines and nitric oxide synthase by the affected astrocytes (Sun *et al.*, 1995). These factors are thought to produce demyelination through cytotoxic effects and recruitment of effector immune cells. Further studies on the mechanisms of virus spread in the primate CNS and the possible relationship between coronavirus infection, cytokine induction, MHC expression and demyelination are needed before attributing a definite role of these viruses in the primate CNS disorders.

Acknowledgements

The work reported here was partially supported by grants NS30530 (Colorado Neurological Institute) and RR00164 (Tulane Regional Primate Research Center) from the National Institutes of Health and by funding from the Rocky Mountain MS Center.

References

- Burks JS, DeVald BL, Jankovsky LD, Gerdes JC (1980). Two coronaviruses isolated from central nervous system tissue of two multiple sclerosis patients. *Science* **209**: 933–934.
- Fleming JO, Wang F-I, Trousdale MD, Hinton DR, Stohlman SA (1993). Interaction of immune and central nervous systems: contribution of anti-viral Thy-1+ cells to demyelination induced by coronavirus JHM. *Regional Immunol* **5**: 37–43.
- Gerdes JC, Klein I, DeVald BL, Burks JS (1981). Coronavirus isolates SK and SD from multiple sclerosis patients are serologically related to murine coronaviruses A59 and JHM and human coronavirus OC43; but not to human coronavirus 229E. *J Virology* **38**: 231–238.
- Lampert PW, Sims JK, Kniazeff AJ (1973). Mechanism of demyelination in JHM virus encephalomyelitis. *Acta Neuropathol* **24**: 76–85.
- Lavi E, Gilden DH, Highkin MK, Weiss S (1984). Persistence of mouse hepatitis virus A59 RNA in a slow virus demyelinating infection in mice as detected by *in situ* hybridization. *J Virol* **51**: 563–566.
- Lowe J, Cox G (1990). In: *Theory and Practice of Histological Techniques*, 3rd edition, Bancroft JD, Stevens A (eds). Churchill and Livingstone, pp 347–348.

- Massa PT, Wege H, ter Meulen V (1986). Analysis of murine hepatitis virus (JHM strain) tropism toward Lewis rat glial cell *in vitro*. Type I astrocytes and brain macrophages (microglia) as primary cell targets. *Lab Invest* **55**: 318–327.
- Murray RS, Cai G-Y, Hoel K, Zhang J-Y, Soike KF, Cabirac GF (1992a). Coronavirus infects and causes demyelination in primates. *Virology* **188**: 274–284.
- Murray RS, Brown B, Brian D, Cabirac GF (1992b). Detection of coronavirus RNA and antigen in multiple sclerosis brain. *Ann Neurol* **31**: 525–533.
- Perlman S, Ries D (1987). The astrocyte is a target cell in mice persistently infected with mouse hepatitis virus, strain JHM. *Microb Pathog* **3**: 309–314.
- Stohlman SA, Weiner LP (1981). Chronic central nervous system demyelination in mice after JHM virus infection. *Neurology* **31**: 38–44.
- Sun N, Grzybicki D, Castro RF, Murphy S, Perlman S (1995). Activation of astrocytes in the spinal cord of mice chronically infected with a neurotropic coronavirus. *Virology* **213**, 482–493.
- Vafai A, Murray RS, Wellish M, Devlin M, Gildea DH (1988). Expression of varicella-zoster and herpes simplex virus in normal human trigeminal ganglia. *Proc Natl Acad Sci USA* **85**: 2362.
- Wang F-I, Stohlman SA, Fleming JO (1990). Demyelination induced by murine hepatitis virus JHM strain (MHV-4) is immunologically mediated. *J Neuroimmunol* **30**: 31–41.
- Watanabe R, Wege H, ter Meulen V (1983). Adoptive transfer of EAE-like lesions from rats with coronavirus induced demyelinating encephalomyelitis. *Nature* **305**: 150–153.
- Weiner LP (1973). Pathogenesis of demyelination induced by a mouse hepatitis virus (JHM virus). *Arch Neurol* **28**: 298–303.
- Weiss SR (1983). Coronaviruses SD and SK share extensive nucleotide homology with murine coronavirus MHV-A59, more than that shared between human and murine coronavirus. *Virology* **126**: 669–677.