

Entry of coronavirus into primate CNS following peripheral infection

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(Received January 19, 1994; accepted in revised form May 2, 1994)

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A previous report demonstrated that intracerebrally inoculated coronavirus produced CNS disease in two species of primates (Murray RS, Cai G-Y, Hoel K, *et al.*, *Virology* 1992; 188: 274–84). We were therefore interested in testing the potential of coronaviruses to infect primate CNS tissue following peripheral inoculation. Four Owl monkeys (*Aotus trivirgatus*) were inoculated intranasally and ocularly and four were inoculated intravenously with coronavirus JHM OMP1 (Murray RS, Cai G-Y, Hoel K, *et al.*, *Virology* 1992; 188: 274–84). Two intranasally and two intravenously inoculated animals received a second intravenous inoculum at 153 days post-infection. The animals were sacrificed 16, 35, 194, and 215 days post-infection. Tissue sections from brain and spinal cord were screened for viral products by *in situ* hybridization and immunostaining. Virus RNA and/or antigen was detected in the brains of all animals and the distribution corresponded to areas of inflammation and edema. Viral products were predominantly found in blood vessels and perivascular regions, suggesting hematogenous spread with entry into the central nervous system through endothelium.

Key words: coronavirus; primate; brain endothelial cells.

Introduction

Coronaviruses are enveloped, positive-sense RNA viruses that produce disease in humans and animals. Depending on the host infected, these pathogens cause peritonitis, enteritis, hepatitis, encephalomyelitis, and respiratory infection. In humans, coronaviruses are associated with upper respiratory infections and probably enteritis.^{2,3} Recently, evidence has been presented that suggests coronaviruses can infect human central nervous system (CNS) tissue.^{4,5}

Surprisingly, the first report of coronavirus RNA and antigen detection in human CNS shows that the viral products are more closely related to murine coronaviruses.⁴ While this is contrary to the presumed species specificity of these viruses data

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showing that the prototypic murine coronavirus, mouse hepatitis virus (MHV) JHM, can replicate, disseminate, and produce CNS disease in two species of primates following intracerebral inoculation¹ support the contention that human CNS can be infected by a 'murine-like' coronavirus. This report also showed that a putative human CNS coronavirus isolate, previously classified as a murine virus, was also capable of producing CNS disease in the two primate species. These reports of coronavirus RNA and antigen detection in human CNS and coronavirus infection of primate CNS were followed by a report of human coronavirus (HCV) 229E RNA detection in human CNS tissue;⁵ 229E is a confirmed respiratory pathogen in humans. Additionally, aminopeptidase N, a glycoprotein expressed on nerve synapse membranes, is a receptor for HCV 229E.⁶ Collectively, these data suggest that the species barrier to coronavirus infection may not be as strict as once thought and that perhaps a diverse range of coronaviruses have the potential to infect human CNS.

Because neurotropic murine coronaviruses can gain access to the murine CNS following peripheral infection⁷⁻¹² and since other known human neurotropic viruses can enter the CNS after primary infection of non-neuronal tissues,¹³⁻¹⁵ we tested the ability of coronaviruses to enter the primate CNS via peripheral routes.

Results

This section is arranged to correspond to the chronology of animal sacrifice, i.e. results from the two animals sacrificed 16 days post-infection (dpi) are described first, followed by the results from the remaining animals sacrificed on 35, 194 and 215 dpi. Figure 1 shows results from the neutralizing antibody assays and Fig. 2 shows representative photomicrographs of *in situ* hybridization, immunohistochemical staining and histology.

Two animals, K183 and K172, inoculated intravenously (i.v.) and intranasally (i.n.), respectively, were sacrificed 16 dpi. Throat swab and blood cultures from K183 and throat, blood, and conjunctiva/corneal swab cultures from K172 taken on 1, 2, 5, 7, and 9 dpi were negative for infectious virus. Neutralizing antibody was not detectable in sera from either animal. Cerebrospinal fluid (CSF) and tissue from brain, spinal cord and lung taken at the time of sacrifice were negative for infectious virus. Pathology showed slight meningitis and mild white matter inflammation in K172 and mild ependymitis, meningitis, choroiditis, and mild white matter edema and inflammation in K183. Viral RNA was not detected in tissue from either animal by *in situ* hybridization. However, viral antigen was detectable in blood vessel endothelium from both animals and in perivascular regions of K183. It appeared that viral antigen was more widely distributed in the intravenously (i.v.) inoculated animal, K183. Sections adjacent to viral antigen positive sections were negative with all control primary antisera (see Materials and methods). Viral products were not detected in trigeminal ganglia or olfactory bulbs from intranasally (i.n.) inoculated animal K172.

Animals K186 and K192, i.v. and i.n. inoculated, respectively, were sacrificed 35 dpi. A throat culture obtained 1 dpi from K192 was positive for infectious virus but all other throat, blood and conjunctiva (K192) cultures from both animals were negative. As with the two animals sacrificed 16 dpi, K186 and K192 never produced detectable neutralizing antibody. While the first four animals sacrificed did not have neutralizing antibody, of the four remaining animals three had detectable levels of neutralizing antibody by 35 dpi. An assay for infectious virus from CSF, brain, spinal cord, and lung tissue was not done for K186 and K192. Mild white matter inflammation in K192 and choroiditis, mild white matter edema, and inflammation in K186 were observed. *In situ* hybridization revealed viral RNA in blood vessel endothelium

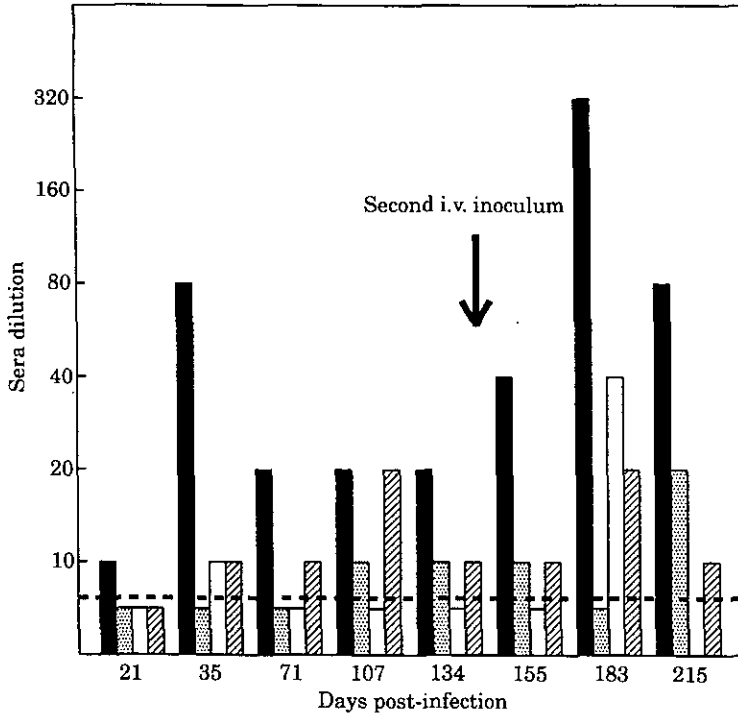


Fig. 1. Neutralizing antibody titers of sera from i.n. and i.v. inoculated Owl monkeys. Results are shown from the four animals sacrificed at later times; the animals sacrificed at 16 and 35 dpi were seronegative. Sera dilutions that produced 50% neutralization of TCID₅₀ are shown; values shown below a 1:10 dilution (dotted line) are considered negative by this method. Neutralizing titer of sera samples taken from all animals before virus inoculation were negative by this criterion. The time of the second virus inoculum (see Results) is indicated on the graph. Animal K179 was sacrificed 194 dpi and therefore no titer is shown for the last time-point. Key: (■) K187 (i.n.); (■) K194 (i.n.); (□) K179 (i.v.); (▨) K181 (i.v.).

[Fig. 2(a)] and occasionally in perivascular regions from both animals. Antigen was again detectable in endothelium and surrounding areas. By comparing *in situ* hybridization with antigen staining results, it appeared that the number of vessels containing viral antigen was greater than the number of vessels positive for viral RNA. As with the two animals sacrificed 16 dpi, viral products were more frequently detected in tissue sections from the i.v. animal, K186, compared to the i.n. animal, K192. Interestingly, viral RNA and antigen were detected predominantly in white matter blood vessels for both animals. Viral products were not detected in trigeminal ganglia or olfactory bulbs from i.n. inoculated animal K192.

While analysis of tissue from the first four animals clearly showed that virus was present in the CNS following peripheral inoculation, the degree of pathology was less than that seen in intracerebrally inoculated Owl monkeys¹ or that described for peripherally infected mice.⁷⁻¹¹ Therefore, we decided to inoculate the four remaining animals, K181 (i.v.), K179 (i.v.), K187 (i.n.) and K194 (i.n.), with a second dose of virus in an effort to produce more dramatic disease. One hundred and fifty-three days after receiving the first inoculum, these animals were inoculated i.v. with additional virus dose.

Assays of neutralizing antibody in sera from these four animals showed they had seroconverted before receiving the second dose of virus. The highest titer was 1:80 for i.n. inoculated animal K187 (35 dpi) and the lowest was 1:10 for i.v. animal K179 (35 dpi) (Fig. 1). To varying degrees, the second inoculum caused a rise in titers.

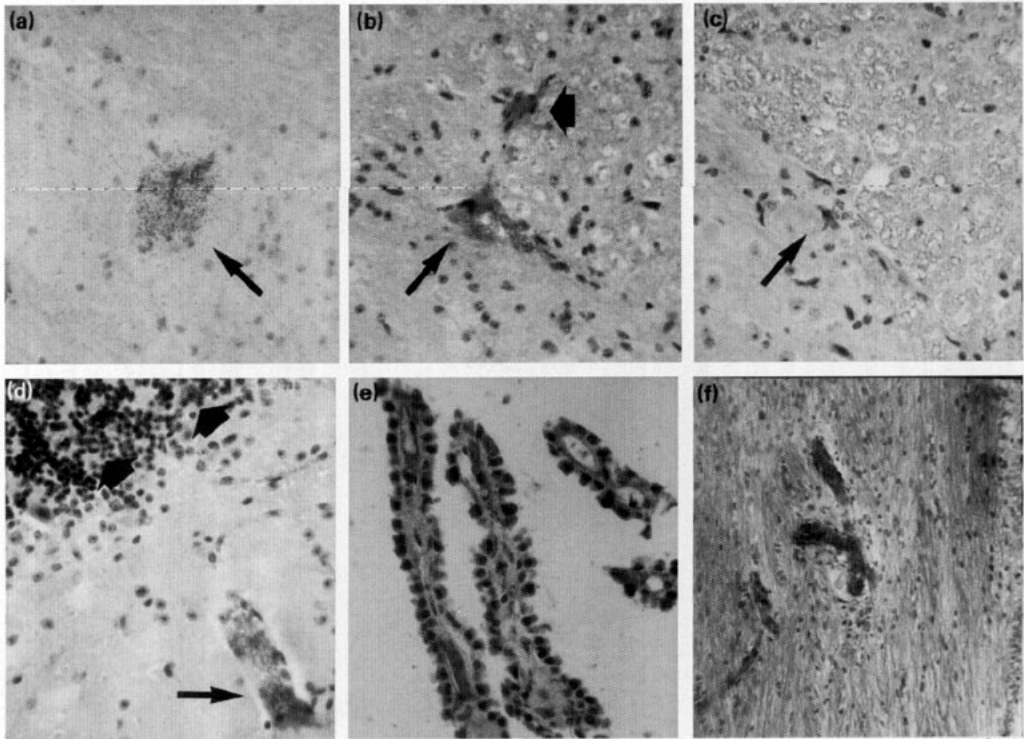


Fig. 2. Detection of viral RNA and antigen in the CNS of peripherally infected Owl monkeys and histochemical staining of infected CNS tissue. (a) Silver grain development over vessel (arrow) in brain white matter tissue from i.n. inoculated animal K192, sacrificed 35 dpi, indicating viral RNA. (b) Positive viral antigen staining in brain from i.v. inoculated animal K179, sacrificed 41 days post-second i.v. inoculation. Arrow and arrowheads indicate staining of vessel and adjacent areas, respectively. (c) Adjacent section to that shown in (b) stained with normal mouse serum as primary antibody; arrow indicates blood vessel. All control primary antisera produced the same result. (d) Viral antigen in cerebellum of animal K179. Arrow and arrowheads indicate staining of vessel and adjacent areas in granular layer, respectively. (e) Viral antigen in choroid plexus of i.n. (+2nd i.v.) inoculated animal K194, sacrificed 62 days post-second inoculation. (f) Luxol-fast blue/periodic acid-Schiff staining of tissue from i.n. (+2nd i.v.) inoculated animal K181, sacrificed 62 days post-second inoculation, showing edema and perivascular cuffing surrounding a blood vessel illustrative of pathology seen in all the virus-inoculated animals. Tissue from sham-inoculated animals (not shown) was processed along with tissue from virus inoculated animals and found to be negative for viral RNA and antigen.

K187 had a titer of 1:320 at 183 dpi. K181 and K194 had a titer of 1:20 at 183 dpi and 215 dpi, respectively (Fig. 1). Blood and throat cultures (and conjunctival cultures from K187 and K194) taken on 1, 2, 5, 7, and 9 dpi (first inoculation) from these four animals were negative for infectious virus. No additional cultures were taken after the second virus inoculum.

On 189 dpi, 36 days after administration of the second dose of virus, animal K179 was observed with hind-limb weakness and tremors; this animal was sacrificed 194 dpi and tissue was processed as before. Infectious virus was not recovered from frozen brain and spinal cord tissue from K179. Immunohistochemical staining showed that this animal had detectable viral antigen not only in the brain but also in the spinal cord. In general, a higher percentage of vessels and perivascular regions were positive for viral antigen in K179 [Fig. 2(b), (d)] when compared to all other animals. *In situ* hybridization analysis was not done on K179 tissues. Pathology showed more widespread white matter edema and inflammation corresponding to the higher number of antigen positive vessels. In addition, this animal also had mild ependymitis, meningitis and choroiditis.

The remaining three animals, K181, K187, and K194, were sacrificed 215 dpi (62 days post-second inoculation). Infectious virus was not recovered from frozen brain tissue from these three animals. Immunohistochemical analysis of tissue from these animals revealed the same results as described above, i.e. viral antigen in vessels and surrounding regions. Figure 2(e) shows viral antigen staining in the choroid plexus of animal K194. Positive antigen staining of these cells was also seen in the other animals. Quantitatively, there appeared to be much less antigen in animal K187 compared to K181 and K194, and tissue analyzed from these three animals showed less antigen when compared to tissue from the animal with neurological dysfunction, K179. *In situ* hybridization analysis was not done on tissues from animals K181, K187 and K194. Despite the second inoculation of virus, the pathology observed in these three animals was comparable to that seen in the first four animals sacrificed [Fig. 2(f)].

Discussion

The results presented in this study show that a primate brain-passaged murine coronavirus is capable of infecting Owl monkey CNS after peripheral inoculation. Viral antigen was found in vessels and perivascular regions which suggests that virus entered the CNS through vascular endothelium. This is consistent with the CNS entry mechanism for many other potential neurotropic viruses. The main route into the CNS during both experimental and natural infection appears to be through vascular endothelial cells (reviewed in Johnson¹⁵). There have been no reports specifically showing MHV infection of brain vascular endothelium infection following peripheral inoculation of mice. However, since murine CNS disease can be induced by peripheral infection,^{8,10,12} non-CNS vascular endothelium can be infected by peripheral inoculation,¹⁶⁻¹⁸ and murine brain endothelial cells can be infected with MHV *in vitro*,¹⁹ it is plausible that CNS endothelium can be infected by hematogenous virus. We have found that MHV JHM can enter Rhesus monkey (*Macaca mulatta*) brain endothelial cells *in vitro* and undergo limited transcription and translation (manuscript in preparation). This supports the results described here which show that virus interaction with endothelium represents a possible entry route into primate CNS.

The finding of virus products in the CNS so long after inoculation indicates that virus infection of CNS vasculature persists over the time periods described here or that virus may be replicating in extra-neural sites then spreading to the CNS. It is reasonable to assume that vascular endothelium in other organs can be infected. This occurs in mice peripherally infected with strains of MHV.¹⁶⁻¹⁸ While we did detect occasional virus antigen-positive areas in sections of liver from i.v. inoculated animal K186 (data not shown), a thorough examination of tissues from other animals was not done for this study. Additional studies are needed to determine the propensity of various strains of coronavirus to infect other primate tissues *in vivo* since these may be a potential reservoir for long-term production of hematogenous virus.

An additional point to make concerning virus replication in the CNS is that, since there was no detection of infectious virus from CNS tissues from any of the animals, it is not certain that active virus replication in the CNS occurred. However, in a previous study, infectious virus could not be cultured from the brains of most intracerebrally inoculated animals,¹ even when abundant viral RNA and antigen were present, indicating there are difficulties rescuing infectious virus from these tissues.

The rationale for inoculation of one-half of the animals by both intranasal and ocular routes was to determine if virus was capable of interneuronal entry into

primate CNS as has been demonstrated in mice.^{7,9,11} Because of the limited number of animals we would not be able to sacrifice at early times and on sequential days in an attempt to detect virus products in olfactory and trigeminal nerves. The intention was to allow the virus, if capable, to establish an acute brain infection through these interneuronal routes. Presumably this infection could then be detected at later times of sacrifice. The lack of detection of virus products in the trigeminal ganglia and olfactory bulbs of i.n. animals K172 and K192 is not a clear indication that virus did not enter the brains by these routes; at the times of sacrifice any active infection of neurons or glia may have ceased and hence no virus products were evident. However, the lack of histopathology in the trigeminal ganglia and olfactory bulbs coupled with the distribution pattern of virus products in the brains of these two i.n. animals suggests that virus did not enter by these routes. Since the pattern of virus RNA and antigen corresponded to that seen in the i.v. inoculated animals, it is most likely that primary virus replication, perhaps in respiratory epithelium, resulted in hematogenous spread to the CNS.

When comparing the results obtained from the i.n. inoculated animals to the i.v. inoculated animals it is difficult to draw any conclusions owing to the small number of animals used in the study. The only apparent difference was that we detected less viral antigen in the two i.n. inoculated animals, K172 and K192, sacrificed 16 and 35 dpi, respectively, compared to the i.v. animals, K183 and K186, sacrificed at the same time points. The detection of infectious virus in the throat culture of i.n. animal K192 and the presence of neutralizing antibody in the i.n. inoculated animals K187 and K194 prior to the later i.v. inoculation suggests that virus replication did occur. Hence the finding of less virus in the CNS of K172 and K192 might be expected if limited primary virus replication in these i.n. animals resulted in a reduced hematogenous virus load compared to that in the i.v. group.

It should be noted that inoculation of the second group of animals, again i.v., at 153 dpi complicates the interpretation of the observations. Since these animals had virus-specific antibodies at the time of the second inoculation, virus-induced pathology may have been altered compared to the pathology observed in the naive animals. While this may have occurred, and in fact may in part explain observations on animal K179 (discussed below), no differences were seen between the animals that received one versus two virus inoculations.

The apparent neurological deficit in animal K179, one of two animals that received two i.v. inoculations, is difficult to explain. The only difference between CNS tissue from K179 and other animals was that more virus antigen was detected in the brain of K179 and antigen was detected in some sections of spinal cord while no antigen was detected in spinal cord from the other animals. Furthermore, the extent of histopathology in the brain and spinal cord of K179 appeared not to account for the neurological deficit. However, it could be postulated that the observed impairment in K179 was due to vasculitis.

Vessel inflammation and necrosis in the CNS is well documented for many animal virus infections, and a few viruses have been implicated in CNS vascular disease in humans.¹³⁻¹⁵ There are reports of coronavirus-induced CNS vasculitis in mice following peripheral infection.^{12,17,18} In these cases of virus-induced vasculitis, vessel wall damage can result from direct virus infection of endothelial cells and/or from immune mediated mechanisms.^{14,15} It is interesting to note that, in addition to the detection of viral products in vascular endothelium and mild perivascular cellular infiltrates shown here, we observed vessel wall necrosis and what appeared to be fibrin deposition in CNS vessels from the group of animals reported here. We also observed similar histopathology in a JHM Omp1 i.n. inoculated Rhesus monkey

from a previous study. CNS tissue from this animal had prominent vessel wall and perivascular necrosis accompanied by cellular infiltration (unpublished data). These observations are consistent with virus-induced vasculitis. The apparent neurological impairment of animal K179 could therefore be explained on this basis as opposed to direct neuron or neuroglia infection.

The methods used for inoculation of the experimental animals in this study clearly do not mimic a natural infection. However, the data show that primate-passaged coronavirus JHM Omp1 can gain access to the CNS of *Aotus trivirgatus* following inoculation by the routes described here. Clearly, more definitive studies are needed to determine if natural respiratory or enteric coronavirus infections of humans result in involvement of the CNS. One step towards achieving this goal would be to collect human coronavirus field isolates and use these to infect primates. This would test the potential of these isolates to cause CNS disease. Concurrently, these same isolates should be used for murine infection to determine if the biological properties of the viruses in this species are similar to those in primates and humans. Key issues to study would be mechanisms of coronavirus dissemination following natural infection in humans, the effects of recurrent infections over many years, modulation of pathogenesis by the immune system, and the propensity of actual field isolates to infect brain vascular endothelia and other CNS cell types.

Materials and methods

Cells and virus. Murine delayed brain tumor (DBT) cells grown in Dulbecco's Modified Eagle's medium, 2–10% fetal bovine serum, penicillin and streptomycin were used for growth of all virus stocks and inocula, for assays of infectious virus from animal tissues and for neutralization assays. Serum virus neutralization assays were performed as follows. Two-fold serial dilutions of sera were mixed 1:1 with virus inocula (~ 100 TCID₅₀/ml) then incubated at room temperature for 1 h; these inocula were then assayed for infectivity on DBT cells grown in 24-well plates. Coronavirus JHM Omp1 was isolated from brain homogenate from a intracerebrally inoculated Owl monkey as previously described.¹ This virus isolate was not plaque purified.

Animals. Four feral adult Owl monkeys (*Aotus trivirgatus*), sedated by intramuscular injection of ketamine (10 mg/kg), were injected with 1 ml of coronavirus JHM Omp1 inoculum into the saphenous vein and four were inoculated with 1 ml virus divided between both nostrils. The nasally inoculated animals also received two drops of virus inoculum in the right eye after corneal scarification. The titer of the inoculum, prepared as previously described,¹ was approximately 10^6 TCID₅₀/ml. Sera samples obtained before virus inoculation and 14, 21, 35, 71, 107, 134, 155, and 183 days post-infection (dpi) or at time of sacrifice were assayed for virus neutralizing antibody as previously described.¹ Throat swab and 2 ml blood samples from the intravenously (i.v.) inoculated animals and throat and conjunctival/corneal swabs and 2 ml blood samples from the intranasally (i.n.) inoculated animals were obtained 1, 2, 5, 7 and 9 dpi and assayed for infectious virus as previously described.¹ One i.v. and one i.n. inoculated animal were sacrificed 16 dpi. Homogenized brain, spinal cord and lung tissue samples and cerebrospinal fluid from these two animals were assayed for infectious virus. Two more animals, one i.v. and one i.n., were sacrificed 35 dpi. On 153 dpi the remaining four animals were re-inoculated i.v. as described above. One animal, K179, was observed to have mild hind-limb tremors and weakness and was sacrificed 195 dpi (42 days post-second inoculation). The remaining three animals were sacrificed 215 dpi (62 days post-second inoculation). For the group of animals receiving the second i.v. inoculation, assays for infectious virus in brain and spinal cords were done using a 10% homogenate of frozen tissue.

Immunostaining of viral antigen, in situ hybridization detection of viral RNA and histochemical staining. Formalin-fixed, paraffin embedded tissues sections (6 μ m) were de-paraffinized and incubated in Ca²⁺/Mg²⁺-free PBS containing 0.25% trypsin at room temperature for 90 min then washed three times in phosphate buffered saline. The trypsin incubation time

was optimized on positive control tissues obtained from intracerebrally inoculated animals.¹ Tissue used for antigen detection was fixed in formalin for no more than 72 h. Monoclonal antibody J.3.1,²⁰ specific for coronavirus nucleocapsid protein, was used as primary antibody. Control primary antisera used were: normal mouse serum, normal rabbit serum and a non-coronavirus specific mouse monoclonal antibody. All primary antibodies were incubated on sections at 4°C for 12–16 h. Secondary antibody was biotinylated rabbit anti-mouse immunoglobulins (Dako) for the mouse primary antibodies or biotinylated anti-rabbit immunoglobulins (BioGenex) for the rabbit primary antibody control. Secondary antibodies were incubated at room temperature for 30 min. Following antibody treatment, tissue was incubated for 20 min at room temperature with alkaline phosphatase-conjugated streptavidin (BioGenex), then chromogen Fast Red TR (BioGenex) was incubated for 20 min at 37°C. Sections were washed in room temperature PBS between each incubation step. Sections were counterstained with hematoxylin. *In situ* hybridization: formalin-fixed, paraffin-embedded tissues containing regions of pathology, unaffected tissues and tissue from a sham-inoculated control animal were analyzed as previously described.^{1,4} The probe used was MHV A59 cDNA clone G344.^{1,21} This cDNA is derived from the nucleocapsid gene of MHV. The gel-purified cDNA insert was labelled with ³²P dATP by the random primer method²² then desalted on Sephadex G-50. Specific activity of the probe was 1–4 × 10⁹ cpm/μg. Specificity of this probe was confirmed by hybridization to MHV JHM infected and uninfected cytopun, fixed DBT cells and to virus-positive brain sections from intracerebrally inoculated animals.¹ Histochemical staining of tissue sections was as previously described.⁴

This work was supported by grants from the Swedish Medical Foundation, the Fausel Foundation and the Nancy Davis Foundation to the Rocky Mountain MS Center and by National Institutes of Health grant RR00164 to the Tulane Regional Primate Research Center.

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