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# Coronavirus Infects and Causes Demyelination in Primate Central Nervous System

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**Two species of primates, Owl and African green monkeys, were inoculated intracerebrally with either the neurotropic mouse hepatitis virus JHM or the putative multiple sclerosis brain coronavirus isolate SD. These viruses caused an acute to subacute panencephalitis and/or demyelination in the infected animals. The course of pathogenesis and sites of detected viral RNA and antigen was dependent both on animal species and virus strain but the results clearly showed that these viruses replicated and disseminated in the central nervous system (CNS) of these primates. This study suggests that human CNS may be susceptible to coronavirus infection.** © 1992 Academic Press, Inc.

## INTRODUCTION

Coronaviruses are ubiquitous animal pathogens that can produce neurologic or systemic illness in infected animals. In humans, coronaviruses are a leading upper respiratory tract pathogen (Hovi *et al.*, 1979) and are associated with gastroenteritis (Resta *et al.*, 1985) but have never been proven to cause serious human illness. However, two reports, one describing the isolation of coronavirus from the brains of two multiple sclerosis (MS) patients (Burks *et al.*, 1980) and the other the electron microscopic observation of a coronavirus in an MS brain perivascular immunocyte (Tanaka *et al.*, 1976), suggest that there may be an association between coronaviruses and human demyelinating disease. There have been no subsequent reports supporting this link between coronaviruses and human demyelination. However, data showing that these viruses produce demyelination (Bailey *et al.*, 1949; Pappenheimer, 1958; Weiner, 1973; Herndon *et al.*, 1975; Lucas *et al.*, 1977; Nagashima *et al.*, 1978; Sorensen *et al.*, 1980; Lavi *et al.*, 1984) and stimulate T-cell mediated autoimmune reactions against CNS antigens in rodents (Stohlman and Weiner, 1981; Watanabe *et al.*, 1983; Kyuwa *et al.*, 1991) suggest that the question concerning the link between coronaviruses and neurologic disease in humans is open.

Intriguing as the animal studies are, human coronaviruses are currently regarded only as respiratory and gastroenteric pathogens simply because they have never been shown to infect human or subhuman primate CNS. In 1956 a report from the German literature stated that intracerebral (IC) inoculation of a 10% homogenate of a mouse hepatitis virus (MHV) JHM-infected mouse brain into *Macaca mulatta* (rhesus) or *Macaca fascicularis* (cynomolgus) monkeys produced an acute panencephalitis (Kersting and Pette, 1956). However, proper analysis to definitively associate JHM with the ensuing encephalomyelitis as opposed to a system contaminate or autoimmune reaction toward inoculated brain antigens was not performed. Additionally, Pappenheimer (1958) commented that several *M. mulatta* monkeys that had been inoculated intracerebrally with JHM by Bailey *et al.* (1949), as described in their original report of JHM-induced neuropathology in rodents, were negative for disease. As such, the role of coronaviruses in subhuman primate or human CNS disease remains uncertain.

We pursued the question of primate CNS coronavirus infection when our analysis of autopsy multiple sclerosis (MS) brain tissue revealed that coronavirus RNA and antigen were detectable in demyelinating plaques and that these viral products were similar to those from prototypic murine coronaviruses. The determination of similarity of these MS coronavirus RNA and antigen products to murine coronavirus products was based on the differential hybridization to cloned cDNA probes derived from various coronaviruses and the use of specific monoclonal antibodies (manuscript submitted). Based on these data we hypothesized that primate CNS is susceptible to neurotropic murine or "murine-like" coronaviruses and decided that this could be tested utilizing current techniques for the analysis of viral infection.

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## MATERIALS AND METHODS

### Viruses and cells

MHV JHM was obtained from Dr. S. Stohlman (Stohlman and Weiner, 1981); this virus was derived from suckling mouse brain homogenates and has been passed in tissue culture in our laboratory without plaque purification. The isolation of coronavirus SD has been described previously (Burks *et al.*, 1980). Both strains of virus were propagated in murine-delayed brain tumor (DBT) cells. These cells were maintained in Dulbeco's modified Eagle's medium (DMEM; GIBCO) containing 5% fetal bovine serum, penicillin, and streptomycin. Infection of DBT monolayers was performed as previously described (Mendelman *et al.*, 1983). The virus inocula were prepared by infecting 75-$cm^2$ flasks of DBT cells with either MHV JHM or coronavirus SD for 16 hr, scraping and pelleting the cells, then suspending each flask's content in 4.0 ml of phosphate-buffered saline (PBS). Infected cell suspensions were titered for infectious virus at the time of inoculation. The suspensions had titers of approximately $10^5$ TCID50/ml.

### Animals and experimental design

Outbred adult Owl monkeys (OMs) and African green monkeys (AGMs), representing New World and Old World primate species, respectively, were inoculated intracerebrally through a burr hole into the right subcortical white matter. Each animal was inoculated with 0.5 ml of a suspension of either coronavirus-infected or uninfected (sham controls) DBT cells. The inoculation procedure was performed under general anesthesia utilizing aseptic conditions by a veterinarian expert in the procedure. The monkeys were observed daily for any clinical signs of infection. Blood (1 ml) was drawn on Days 2, 7, 10, 14, 21, and 35 postinoculation (DPI) and assayed for infectious virus as follows. Blood specimens were placed in EDTA tubes and 10-fold dilutions from $10^{-1}$ to $10^{-4}$ of the whole blood were made in DMEM containing 5% fetal bovine serum. The diluted blood samples were inoculated into triplicater wells of 24-well culture plates seeded with DBT cells Cells were observed microscopically for viral cytopa thology. Virus neutralizing antibody titers of sera were determined for all monkeys prior to inoculation and then 7, 14, 21, 35, 60, and 90 days postinoculation or at time of sacrifice. Sacrifice occurred when neurologic signs became evident or at scheduled 90 or 120 DPI endpoints. Brain tissue from the inoculated right cerebrum and cerebrospinal fluid (CSF) from each animal was tested at the time of sacrifice for infectious virus as follows. Brain tissue was homogenized in a glass tissue grinder then clarified by centrifugation. Dilutions in DMEM ($10^{-1}$ to $10^{-4}$) of the clarified homogenate or of the CSF were plated onto DBT cells. These were examined for viral cytopathology.

### *In situ* hybridization, antigen detection, and histochemical staining

The probe used for *in situ* hybridization was MHV A59 cDNA clone G344 (Budzilowicz *et al.*, 1985). Insert cDNA was excised from the plasmid, gel purified, and labeled with [$^{32}$P]dATP by the random primer method (Feinberg and Vogelstein, 1983) then desalted; specific activity of the probe was $1-4 \times 10^9$ cpm/$\mu$g. Specificity of this probe was confirmed by hybridization to MHV JHM or coronavirus SD-infected and uninfected fixed DBT cells. Primate tissue used for *in situ* hybridization was either frozen or formalin-fixed at the time of an animal's sacrifice. Hybridization of sections was performed as described (Vafai *et al.*, 1988). Briefly, tissue was hybridized at 42° for 24 hr then washed at 37° for 48 hr with 50% formamide, 10 m*M* Tris, pH 7.2, 1 m*M* EDTA, 0.6 *M* NaCl. Sections were then dehydrated in graded alcohols containing 0.3 *M* ammonium acetate, air dried, coated with NTB-2 photographic emulsion (Eastman Kodak Co., Rochester, N.Y.), air dried, and placed in a desiccated container at 4°. Emulsion was developed after 3 to 5 exposure days and then the tissue was counterstained with hematoxylin and eosin. Microscopic examination of slides was done with both brightfield and darkfield optics. Pretreatment of tissue sections from infected animals or control infected cells with RNase abolished positive hybridization.

*Antigen detection.* Frozen sections were fixed in −20° methanol for 10 min then washed three times with PBS. Sections were incubated with monoclonal antibody (Mab) J.3.1 (Fleming *et al.*, 1988) at 37° for 20 min then washed with PBS. Immunoperoxidase detection was done with Mab J.3.1 utilizing the Peroxidase Anti-Peroxidase kit from BioGenex Laboratories. Pathologic examination of brain tissue was performed after staining of sections with hematoxylin and eosin or luxol-fast blue/periodic acid-Schiff. Tissue sections from the sham-inoculated animals were analyzed for viral RNA and antigen concurrently with those from infected animals. The regions of the CNS from which the tissue was selected and the number of sections analyzed per region were the same for the sham- and virus-inoculated animals.

### Western, Northern, and PCR analysis

All of the primate tissue used for Western, Northern, or PCR analysis was frozen at −70° immediately after removal from the animals at time of sacrifice.

*Western blot analysis.* Tissues and control-infected

DBT cells were homogenized in 0.1 *M* Tris–Cl, pH 7.0, 2% SDS, and 0.1 *M* DTT. The samples were boiled for 5 min and centrifuged in 1.5 ml eppendorf tubes for 20 min. The supernatants were electrophoresed on a 10% SDS–polyacrylamide gel then transferred to nitrocelluse paper. The blot was placed in Tris-buffered saline (TBS; 50 mM Tris–Cl, pH 7.5, 150 m*M* NaCl), 3% BSA for 1 hr at room temperature and then incubated with the Mab J.3.1 for 1 hr at room temperature. The antibody was removed and the blot washed with TBS, 0.2% Triton X-100, 0.2% sodium deoxycholate, and 0.1% SDS for 40 min with gentle agitation. The blot was then incubated for 1 hr at room temperature with horseradish peroxidase conjugated goat anti-mouse IgG (Bio-Rad) that had been diluted 1000-fold with TBS–3% BSA, followed by a wash as described above. The substrate solution was prepared by dissolving 30 mg of 4-chloro-1-naphthol (Aldrich Chemical Co., Inc.) in 10 ml ice-cold methanol prior to mixing with 50 ml TBS containing 30 $\mu$l of 30% hydrogen peroxide. The chromogenic reaction was conducted by placing the blot into the substrate solution for 3–5 min.

*Northern blot analysis.* Total RNA was extracted from tissues and control-infected DBT cells as described (Chomczynski and Sacchi, 1987). RNAs were electrophoresed on a 1% glyoxal agarose gel then transferred to Zeta-Probe membranes (Bio-Rad). Blots were probed with a $^{32}$P random primer labeled, gel-purified insert from cDNA clone G344. Hybridization and washing conditions were as recommended by the manufacturer of the membrane.

*PCR detection of coronavirus in brain.* Total RNA extracted from tissues was reversed transcribed using the following reaction conditions. One microgram of RNA was mixed with 100 pmole of random hexamers (Boehringer-Mannheim) in a total volume of 15 $\mu$l, heated at 65° for 5 min, then cooled to room temperature. Ten microliters of a enzyme/buffer/dNTP mix was added to the RNA/primer tube and the reaction was incubated at 43° for 1 hr. The final reverse transcriptase reaction conditions were 40 $\mu$g/ml RNA, 4 $\mu$*M* random hexamers, 50 m*M* Tris, pH 8.3, 100 m*M* KCl, 10 m*M* $MgCl_2$, 4 m*M* DTT, 1 unit/$\mu$l RNasin (Promega), 1 m*M* dNTPs, and 5 units of AMV reverse transcriptase (LifeSciences, Inc.). Reactions were heated at 97° for 5 min and then stored at −20°. PCR reaction conditions were as follows. Tubes containing 85 $\mu$l of buffer, primers, enzyme, and approximately 100 $\mu$l of mineral oil were heated to 80° in the Perkin–Elmer Thermocycler. Five microliters of the cDNA from the reaction described above was mixed with 10 $\mu$l of 2 m*M* dNTPs in a separate tube and this was then added through the oil into the PCR reaction tube. The final reaction conditions were: 10 m*M* Tris, pH 8.8, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, 0.1% Triton X-100, 0.5 $\mu$*M* of each primer, and 5 units of Taq polymerase (Promega). The temperature profile for the reactions was as follows: 95°/1 min, 58°/1.5 min, 72°/3 min for 3 cycles then 95°/1 min, 62°/1 min, 72°/2 min for 36 cycles followed by incubation at 72° for 7 min. The sequences of the PCR primers, CVP3 (5′IIAAATTGCTIITCTTGTTCTGGC; I = inosine) and CVP4 (5′CCAAAATTCTGATTAGGGCCTCTC), used for these reactions were based on the published sequences of the nucleocapsid genes of MHV A59 and human coronavirus (HCV) OC43 (Armstrong *et al.*, 1983; Kamahora *et al.*, 1989). Inosine residues were inserted into CVP3 due to the lack of sequence identity between MHV and OC43 at these four positions; base pairing between inosine and adenosine or cytosine allows this primer to have only two mismatches whether annealed to MHV or OC43 target sequences. PCR amplification with CVP3 and CVP4 will produce a 186-bp product from either MHV A59/JHM- or HCV OC43-derived cDNA. These primers span a region extending from nucleotide 858 to 1043 or 784 to 969 for MHV A59 or HCV OC43, respectively. The PCR reaction products were analyzed by gel electrophoresis and Southern blot hybridization using Nytran membranes (Schleicher and Scheull). Hybridization (performed at 55°) and washing conditions were as recommended by the manufacturer. The $^{32}$P-5′-labeled probe used for hybridization, CVPP, has the sequence 5′AAGCAIAITGCCAAAIAAGTCAGICAGAAAATTTT.

## RESULTS

We found that OMs and AGMs were susceptible to infection by coronavirus JHM and SD with important differences between virus strains and primate species. Neutralizing antibody titers (50% neutralization of TCID50) of sera were determined before inoculation and 7, 14, 21, 35, 60, and 90 days postinoculation (DPI) or at time of sacrifice for each virus-infected and control animal. Neutralizing titers were not determined for two animals, K199 and K209, at the time of sacrifice (120 DPI); the last determination was performed 90 DPI for these two animals. The four OMs, inoculated with either JHM or SD, and two of the AGMs, K199 and K209, inoculated with SD and JHM, respectively, had detectable neutralizing antibody titers (Fig. 1). The two OMs, K191 and K063, that received the brain passaged JHM, JHM OMp1, had negative antibody titers but these animals were sacrificed at only 10 and 12 DPI, respectively. All blood samples, drawn at 2, 7, 10, 14, 21, 35, and 60 DPI or at time of sacrifice, were negative for infectious virus; CSF samples taken at time of sacrifice were negative for infectious virus. Only one animal, OM K177, receiving tissue culture passaged virus (JHM) had recoverable infectious virus in

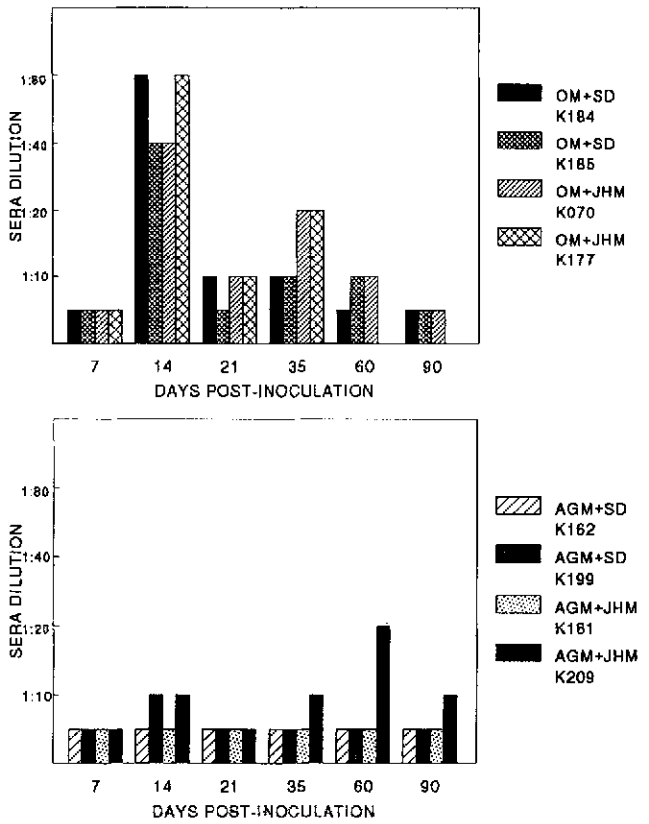



FIG. 1. Neuralizing antibody titer of sera from MHV JHM and coronavirus SD-inoculated monkeys. The top and bottom panels show results from Owl monkeys (OM) and African green monkeys (AGM), respectively. Two animals from each species group were inoculated with either SD or JHM. Sera samples were taken on the days indicated; dilutions that produced 50% neutralization of $TCID_{50}$ are shown. Values shown below a 1:10 dilution are considered negative by this method; all samples taken from animals before virus inoculation or taken from sham-inoculated animals during the course of this study were negative by this criterion. OM K177 (upper panel) was sacrificed 35 days postinoculation when signs of neurological dysfunction became evident. Neutralizing titers for the two OMs that received the brain passaged virus, JHM OMp1 (see text and Table 1), are not shown here; both of these animals were seronegative by this method at time of sacrifice.

the brain at the time of sacrifice. The two monkeys, OMs K191 and K063, inoculated with this brain passage isolate also had infectious virus in assayed brain tissue. Assays for viral RNA by PCR, Northern blot analysis or *in situ* hybridization and viral antigen by Western blot analysis, or immunohistochemical staining was done on extracted material or tissue sections utilizing frozen or formalin fixed tissue. Freezing or fixation of tissues was done immediately after each animal was sacrificed at the designated time point. The results of RNA and antigen detection for each animal is discussed below. Table 1 summarizes neutralizing antibody assays, results of virus isolation from blood, brain, or cerebral spinal fluid, viral RNA and antigen analysis, and pathology for each animal. Note that one animal, OM K185, that received coronavirus SD (Fig. 1) is not included in Table 1; this animal has not been sacrificed at this time. This animal also had no detectable virus in blood samples drawn at the times indicated above.

### Infection of Owl monkeys with MHV JHM

JHM-infected OMs K177 and K070 were sacrificed 35 and 90 DPI, respectively. OM K177 was sacrificed at this earlier time because of observed tremors. JHM caused an acute panencephalomyelitis with grey and white matter inflammation in both of these animals. Associated demyelination was observed by the luxol-fast blue/periodic acid-Schiff staining method in areas of white matter inflammation. Phagocytic cells containing digested myelin were observed by electron microscopy in these areas of demyelination (Fig. 2H); recognizable virus was not seen by ultrastructure. Viral RNA and antigen were detected in both grey and white matter by *in situ* hybridization and immunofluorescence, respectively. RNA extracted from OM K070 CNS was also assayed by PCR and the expected coronavirus specific amplified product was observed (Fig. 4C). Virus was recovered from the brain of one OM (K177) infected with JHM. This isolate, designated JHM-OMp1, upon passage by IC inoculation into two naive and healthy OM (K063 and K191) caused severe disseminated encephalomyelitis by 10 to 12 DPI demonstrating rapid adaptation to the primate system; these animals were sacrificed at this time due to clinical signs of disease. JHM virus could be recovered (designated JHM-OMp2) from the brains of both of these OMs and abundant amounts of viral products were demonstrable in the brains of both animals (Figs. 2A–2C, 3F, and 4A–4C). JHM-OMp1 caused a shift toward more severe inflammatory changes in white matter areas (Figs. 3G and 3H) compared to the original JHM-infected OM.

### Infection of African green monkeys with MHV JHM

In contrast to the OMs, JHM-infected AGMs K161 and K209, sacrificed at 90 and 120 DPI, respectively, appeared healthy at the time of sacrifice. Virus could not be recovered from brain, blood, or CSF and the antibody response was minimal (Table 1). Histopathology demonstrated mild meningitis and a few areas of white matter inflammation with associated edema and demyelination (Figs. 3B and 3C). Viral RNA was detected in both animals by *in situ* hybridization in irregularly defined cells in these white matter areas (Fig. 2G). The number of cells in the white matter containing viral RNA was lower than the number seen in the JHM-infected OMs. Viral RNA was not detectable in grey mat-

TABLE 1

SUMMARY OF PRIMATE PATHOGENESIS

| Animals | DPI | Ab* | Viral cultures | | | Viral RNA | | | Viral antigen | | Pathology H&E/LFB |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Bld | Br | CSF | PCR | North | ISH | IH | West | |
| OM + SD K184 | 90 | 1:80 | – | – | – | + | – | + wm cell & choroid meninges | – | – | wm demyelination & meningitis |
| OM + JHM K177 | 35* | 1:80 | – | + | – | ND | ND | + grey & wm cells | + | ND | Severe encephalomyelitis |
| OM + JHM K070 | 90 | 1:40 | – | – | – | + | – | + grey & wm cells | + | ND | Mild encephalomyelitis |
| OM + SHAM K175 | 90 | – | – | – | – | – | – | – | – | – | Normal |
| OM + JHM-OMP1 K191 | 10* | – | – | + | – | ND | + | + grey & wm cells | + | + | Severe encephalomyelitis |
| OM + JHM-OMP1 K063 | 12* | – | – | + | – | ND | + | + grey & wm cells | + | + | Severe encephalomyelitis |
| AGM + SD K162 | 90 | – | – | – | – | ND | – | + wm cells | – | – | wm inflammation, reactive astrocytes |
| AGM + SD K199 | 120 | 1:10 | – | – | ND | – | – | – | – | – | wm inflammation, reactive astrocytes |
| AGM + JHM K161 | 90 | – | – | – | – | + | – | + wm cells | – | – | wm inflammation, mild |
| AGM + JHM K209 | 120 | 1:20 | – | – | ND | ND | – | + wm cells | – | – | wm inflammation, mild |
| AGM + SHAM K094 | 90 | – | – | – | – | ND | – | – | – | – | Normal |

Note. OM, Owl monkey; AGM, African green monkey; JHM, tissue culture passage virus; SD, putative MS brain isolate; JHM-OMP1, virus recovered from K177; DPI, days postintracerebral inoculation that animal was sacrificed; *, sacrificed when animal appeared ill; Ab, neutralizing antibody titers. Sera assayed 7, 14, 21, 35, 60, and 90 DPI or at time of sacrifice. Value noted is highest level observed. See Fig. 1 for time course. A negative response was <1:10; +, positive; –, negative; Bld, blood. Samples were assayed for infectious virus 2, 710, 14, 21, and 35 DPI; Br, brain. Fresh homogenates were assayed for infectious virus at time of sacrifice; CSF, cerebrospinal fluid. Samples were assayed for infectious virus at time of sacrifice; ND, not done; PCR, polymerase chain reaction assay for viral RNA extracted from brain; North, Northern analysis for viral RNA extracted from brain; ISH, *in situ* hybridization for viral RNA. See text for description of positive areas; IH, immunohistochemistry for viral antigen; West, Western blot analysis for viral proteins extracted from brain; wm, white matter; H&E/LFB, hematoxylin & eosin/luxol-fast blue staining.

ter areas. RNA from only one of the animals, K161, was assayed by PCR; this yielded a positive result (Fig. 4C). Viral antigen was not detectable by immunoperoxidase staining.

### Infection of Owl and African green monkeys with coronavirus SD

Both OMs and AGMs infected with the putative MS coronavirus isolate SD appeared healthy throughout the experimental time period of 90 to 120 DPI. Virus could not be cultured from either OM or AGM brains. Pathologic changes in the AGMs, K162, and K199, sacrificed at 90 and 120 DPI, respectively, were very similar to those observed for JHM-infected AGMs. The pathologic changes consisted of focal areas of mild demyelination (Fig. 3A), mild periventricular white matter inflammation, perivascular inflammation, and foci of white matter vacuolation similar to that seen in OM K184 infected with SD (Figs. 3D and 3E). OM K184 was sacrificed 90 DPI. Similar to the JHM-infected AGMs, viral RNA was detectable by *in situ* hybridization only in white matter cells from SD-infected AGMs. Viral antigen was not detected.

SD RNA could be detected by *in situ* hybridization in cells of the arachnoid meninges, choroid plexus in addition to white matter in the infected OM K184 (Figs. 2D–2F). Meningeal or choroid cells in AGMs or JHM-infected OM and sham-inoculated monkeys were negative for viral RNA. Interestingly, in OM K184 no inflammation was observed in the choroid plexus indicating a lack of significant viral protein expression at this site. Viral RNA was also detectable in the brain of OM K184 by PCR (Fig. 4C) but not by Northern analysis; antigen could not be detected.

## DISCUSSION

The results presented in this report show that coronaviruses can replicate and disseminate in primate CNS. The two viruses used in this study, MHV JHM and SD, are classified as a prototypic murine coronavirus and murine-"like," respectively. As with other families of viruses, coronaviruses have been divided into

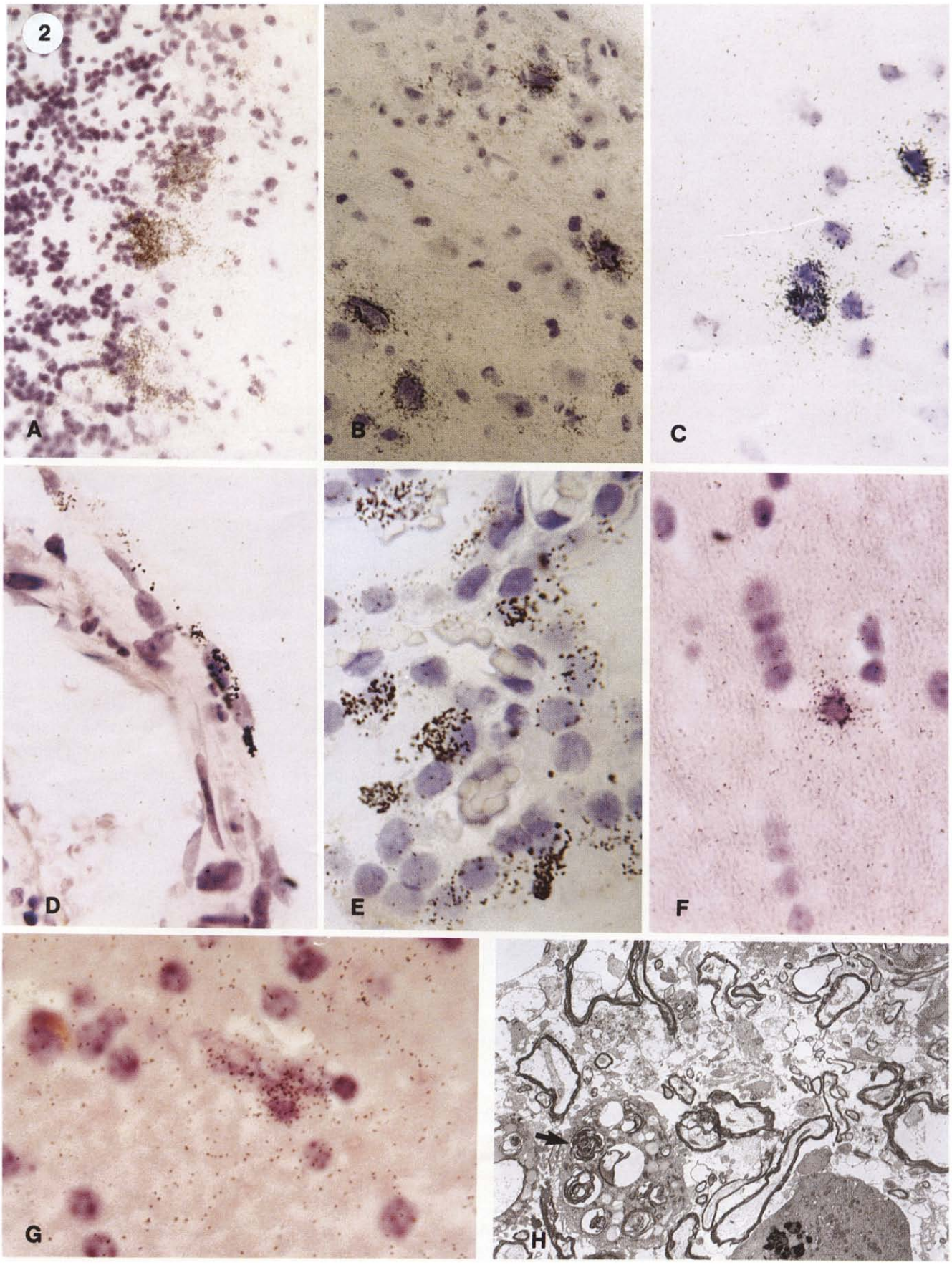
groups based on antigenic cross-reactivity, nucleic acid sequence identity, and/or species of host infected. Reports on *in vitro* infectivity of coronaviruses show that some viruses are promiscuous in their species specificity. Bovine coronavirus (BCV) infection of human (HRT) and canine (MDCK) cell lines (Deregt *et al.*, 1987), porcine coronavirus hemagglutinating encephalomyelitis virus (HEV) infection of a canine (MDCK) cell line (Schultze *et al.*, 1990), canine coronavirus (CCV) infection of a fetal cat cell line (Shockley *et al.*, 1987), and human coronavirus OC43 infection of primary cultures of mouse neural cells (Pearson and Mims, 1985) are some examples of *in vitro* cross-species infection. Although these results demonstrate that some coronaviruses can infect cell types of a species different from the natural host and while there are examples of natural cross-species infection in other virus families, very little is known about coronavirus species specificity as it relates to natural hosts. The data shown here suggest that the species infectivity barriers for some coronaviruses may not be as strict as once thought.

Despite this question of species specificity, there are similarities between infection of primates, as described here, and infection of rodent CNS. First, passage of MHV JHM in primate CNS resulted in isolation of virus, JHM-OMp1, that produced a more severe disease in a shorter time period when compared to the original virus inoculum. This suggests changes in virulence and/or CNS cell tropism in this primate brain isolate. Passage of MHV JHM and related coronaviruses in rodent brain also yields virus populations with altered disease spectrums and is thought to be attributable to mutations in specific viral genes, most notably the spike glycoprotein (Taguchi *et al.*, 1986; Makino *et al.*, 1987; Morris *et al.*, 1989). We are now analyzing various regions of the JHM-OMp1 genome in an attempt to determine what mutations may be responsible for this isolate's altered virulence. Second, viral RNA was demonstrable in some infected primates without production of detectable antigen (see Table 1, AGMs K162, K161, and K209 and OM K184). Perhaps this was due to sampling error or to limits of the antigen detection method but *in situ* hybridization analysis during the chronic phase of MHV A59 or JHM murine CNS infection reveals cells that contain viral genome at a time when viral antigen expression is not detected (Mendelman *et al.*, 1983; Lavi *et al.*, 1984; Sorensen *et al.*, 1985). Third, virus replication, dissemination and accompanying pathology was evident in the infected primates and yet infectious virus could only be isolated from one animal that had received the tissue culture passed virus. This type of nonproductive infection, i.e., lack of recoverable infectious virus, and disease has been observed in mice during the chronic phase of infection that were inoculated with MHV A59 and JHM and related viruses (Stohlman and Weiner, 1981; Mendelman *et al.*, 1983; Lavi *et al.*, 1984). However, a thorough time course study needs to be done with the primates to define the relationship between production of infectious virus and progression of disease. Fourth, coronavirus SD RNA could be detected by *in situ* hybridization in cells of the arachnoid meninges, choroid plexus in addition to cells of the white matter. This is similar to MHV3 infection in mice since virus can also be found in these diverse cell types (Tardieu *et al.*, 1986). These sites of infection indicate a possible CSF route for CNS dissemination of virus. These similarities between primate CNS infection and the rodent system suggest that the mechanisms for virus replication, dissemination, and pathogenesis are not species dependent.

Surprisingly, the viruses used in this study had been maintained in murine cells and did not require special

FIG. 2. *In situ* hybridization studies showing cellular distribution of JHM or SD viral RNA and electron microscopy showing demyelination. Positive signal is demonstrated by silver grain (black) development. Sections were stained with hematoxylin or hematoxylin & eosin. (A) Positive hybridization signal in cytoplasm of cerebellar purkinje cells in OM K063 infected with JHM-OMp1. Magnification, 490×. (B) OM K063 with many cortical neurons showing evidence of infection. Magnification, 490×. (C) OM K191 infected with JHM-OMp1 demonstrating positive cytoplasmic hybridization in white matter cells. Magnification, 785×. (D) OM K184 infected with coronavirus SD showing cytoplasmic viral RNA in arachnoid meningeal cells. Note where this membrane covers a subarachnoid blood vessel. Magnification, 785×. (E) Choroid plexus cells in lateral ventricle of OM K184 has abundant cytoplasmic hybridization signal. Magnification, 785×. (F) A paraventricular white matter cell of OM K184 contains viral RNA. Magnification, 785×. (G) An irregularly shaped cell in white matter of JHM-infected AGM K161, most likely a microglial cell, has detectable viral RNA. Similar finding were observed for SD-infected AGM. (H) EM of white matter inflammation in JHM infected OM K177. Arrow shows phagocyte processing myelin membranes in lysosomes. No viral particles were observed. Magnification, 2155×.

FIG. 3. Histopathology of primates infected with coronaviruses. (A) Cerebellar peduncle in SD-infected AGM K162 showing patches of demyelination as evidenced by lack of luxol-fast blue/periodic acid-Schiff (LFB/PAS) blue staining. Area also is hypercellular. Magnification, 110×. (B) Periventricular area of white matter vacuolation in AGM K209 infected with JHM. LFB/PAS staining. Magnification, 360×. (C) White matter inflammation with associated hypertrophic astrocytes in JHM-infected AGM K161. LFB/PAS staining. Magnification, 720×. (D) Periventricular white matter adjacent to area shown in E showing vesicular changes in SD-infected OM K184. LFB/PAS staining. Magnification, 360×. (E) H&E staining of subependymal perivenule cuff of inflammatory cells in SD-infected OM K184. Magnification, 720×. (F) Acute cortical grey matter inflammatory infiltrate in JHM-OMp1 infected OM K191 stained for viral antigen. Note brown reaction product in cells and axons (arrows). (G) Acute perivascular cuff in JHM-OMp1-infected OM K063 with cells containing myelin fragments evident. LFB/PAS staining. Magnification, 1350×. (H) Same as for G showing acute perivenular inflammatory cuffs in white matter.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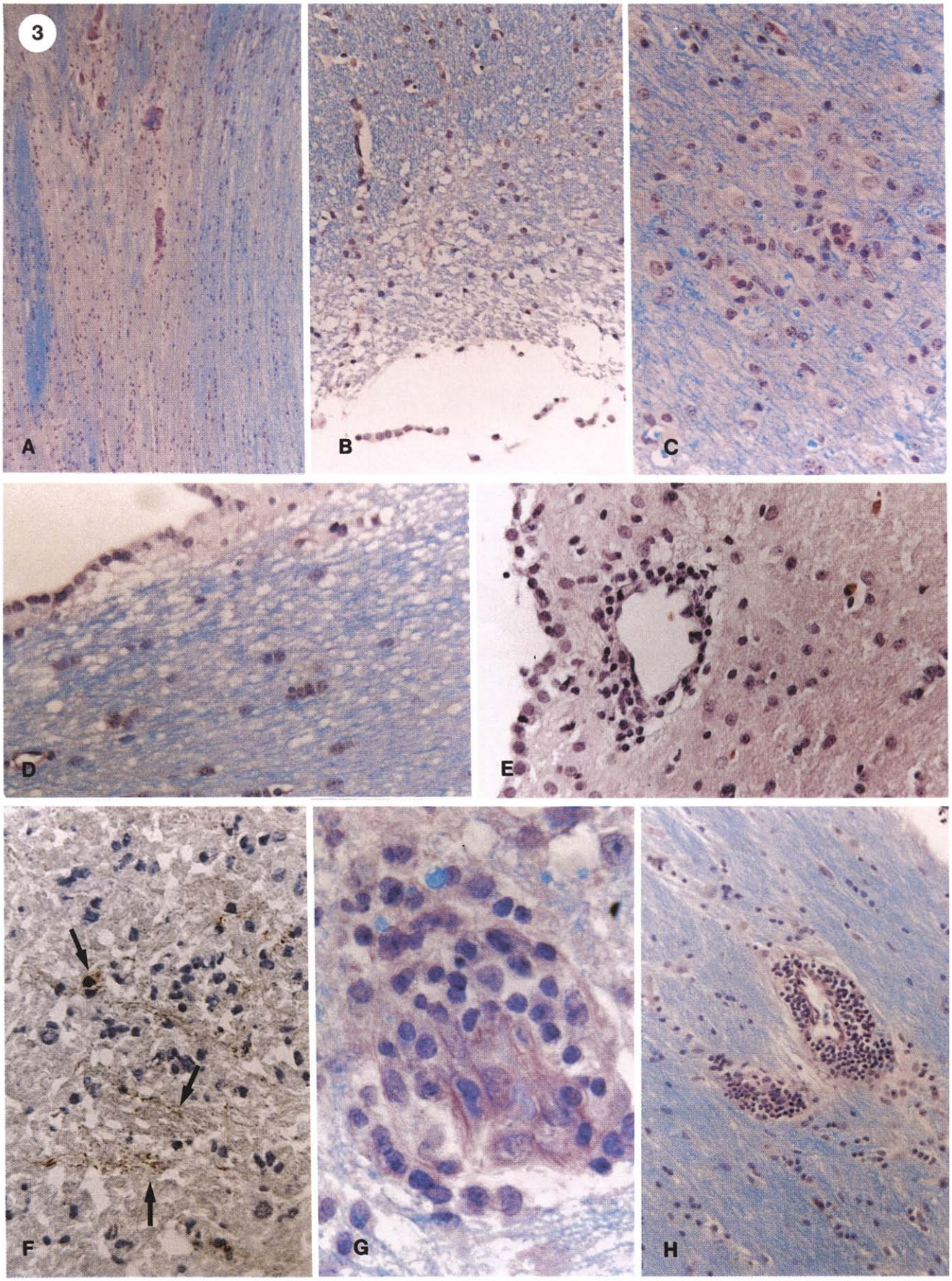
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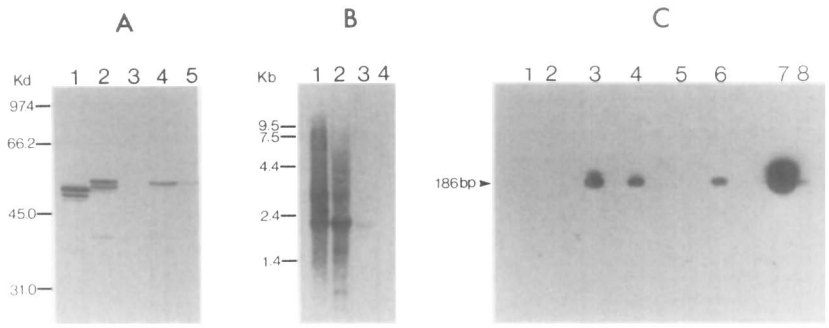


FIG. 4. Western, Northern, and PCR analysis of proteins and RNA from primate brains. (A) Western blot of proteins extracted from: lane 1, coronavirus SD-infected DBT cells; lane 2, MHV JHM-infected DBT cells; lane 3, sham-inoculated OM, K175; lane 4, MHV JHM2-inoculated OM, K191; lane 5, MHV JHM2-inoculated OM, K063. The antibody used for detection of nucleocapsid protein was Mab J.3.1. (B) Northern blot of RNA extracted from: lane 1, coronavirus SD-infected DBT cells; lane 2, MHV JHM-OMp1-inoculated OM, K063; lane 3, MHV JHM-OMp1-inoculated OM, K191; lane 4, sham-inoculated OM, K175. The probe used for hybridization detection of viral RNA was a gel-purified insert from clone G344. (C) PCR analysis of RNA extracted from: lane 2, sham-inoculated OM, K175; lane 3, MHV JHM-inoculated AGM, K161; lane 4, coronavirus SD-inoculated OM, K184; lane 5, coronavirus SD-inoculated AGM, K199; lane 6, MHV JHM-inoculated OM, K070; lane 7, HCV OC43-infected mouse brain homogenate; lane 8, coronavirus SD-infected DBT cells. Lane 1 shows a negative control reaction that had no cDNA added.

adaptation to primate systems prior to infection. This fact, and the demonstration in this study that CNS infection was observed in outbred new and old world primates, implies that CNS susceptibility to coronavirus infection is genetically conserved among primates and between rodents and primates. Since a primary determinant of virus infectivity for a particular cell type is at the level of virus entry into that cell the data in this report imply that at least primate and rodent CNS cell populations share a conserved receptor or mechanism for virus entry. This is consistent with our unpublished data showing that dissociated human brain cells and the human astrocytoma cell line STTG (Barna *et al.*, 1985) have binding receptors for coronavirus SD and MHV A59. It should be mentioned at this point that both viruses used in this study were not tested for either hemagglutinating or esterase activity or expression of the hemagglutinin-esterase glycoprotein. In light of recent data that show that this virion polypeptide may be a determinant that influences infectivity and/or pathogenicity of coronaviruses, this will be an important question to address as it relates to the primate CNS disease described here. It will be interesting to determine the susceptibility of primate CNS to other coronaviruses in order to define any relationship between infection and expressed virion proteins.

One of the viruses used in this study, coronavirus SD, and another isolate, SK, were reportedly recovered from brain tissue obtained from MS patients at autopsy (Burks *et al.*, 1980). These two virus isolates have serologic cross-reactivity to both murine coronaviruses and HCV OC43 (Gerdes *et al.*, 1981) but others have demonstrated that they are antigenically and genetically more closely related to murine coronaviruses than to prototypic human coronaviruses (Fleming *et al.*, 1988; Weiss, 1983). Our unpublished data show a 97% sequence identity in the 3′ end of the genome between SD and MHV JHM in agreement with these earlier reports. These data indicate that the putative MS viruses may represent contaminants derived from the murine system used for their isolation or perhaps that they are recombinants derived from human and murine coronaviruses. However, in light of the results discussed in the present study, it is possible that coronaviruses, similar to the prototypic murine viruses, can infect the CNS of humans.

If the assumption is made that coronaviruses can replicate and produce disease in human CNS then one must ask if these viruses can gain access to the CNS by natural routes of infection. This question has been investigated in the rodent system and the results show that murine coronaviruses may enter the CNS from peripheral trigeminal or olfactory nerves after intranasal inoculation (Bailey *et al.*, 1949; Barthold *et al.*, 1986, 1990; Lavi *et al.*, 1986; Perlman *et al.*, 1989, 1990). Conjunctival inoculation leads to an uveitis and subsequent transport of coronaviruses into the CNS via the optic nerve (S. G. Robbins, personal communication). These studies have shown that coronaviruses may gain CNS access through nonhematogenous routes obviating the need to cross the blood–brain barrier. We are currently testing the hypothesis that coronaviruses can reach the CNS of primates following intranasal or conjunctival inoculation.

Additional investigation of subhuman primate CNS coronavirus infection should provide some insight into potential mechanisms of CNS disease in humans. The use of primates as a model system for human CNS coronavirus infections will eliminate some uncertainties that arise when trying to extrapolate *in vitro* results to *in vivo* infectivity questions. In addition, the results presented here suggest that the murine coronavirus

system may be more relevant to human disease than has previously been thought. The primate system may provide a vehicle by which conclusions drawn from the MHV mouse and rat models of CNS infection can be tested in a species more closely related to humans. Therefore, more work is needed in other coronavirus systems and especially in the primate system to determine if coronaviruses induce or contribute to CNS disease in humans and to learn about the mechanisms of pathogenesis.

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