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In Vivo and in Vitro Models of Demyelinating Disease

IX. Progression of JHM Virus Infection in the Central Nervous System of the Rat during Overt and Asymptomatic Phases

O. SORENSEN, M. B. COULTER-MACKIE, S. PUCHALSKI, AND S. DALES

Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada

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JHM virus, when inoculated into neonatal rats, can cause either a rapidly fatal acute encephalomyelitis or, after longer incubation periods, a paralytic disease. The cerebrospinal fluid (CSF) and serum anti-JHM virus IgG concentrations present in rats prior to onset of clinical symptoms or during the acute and paralytic phases of disease were compared. High CSF/serum ratios, indicative of local antibody production in the CNS, were noted only where disease was demonstrable suggesting that local antibody production accompanied the infection but did not prevent the neurological disease. Among animals in which neurologic symptoms had not become manifest, only those with elevated CSF/serum ratios were found to have histological CNS lesions. Immunofluorescent microscopy indicated that viral antigens were present in both glia and neurons. Antigen-positive cells were frequently present in histologically normal CNS tissue, while regions of necrosis were antigen negative. Testing for the presence of viral RNA with JHM cDNA probes revealed that the virus was rapidly disseminated throughout the CNS, presumably establishing centers of infection prior to the development of recognizable tissue damage. Viral RNA was also detected in the CNS following recovery from paralysis and as late as 5 months postinfection, where no disease occurred. These findings indicate that, although infection by JHM virus can spread rapidly throughout the CNS, formation of lesions during chronic disease is a slower process. The current data and previous observations suggest that JHM virus can remain in a latent state for periods of at least several months in rats without apparent neurologic disease despite the absence of any known provirus phase in the replicative strategy of coronaviruses.

INTRODUCTION

Several strains of murine coronaviruses, including MHV-A59, MHV-3, and JHM, are neurotropic in rodents and elicit either an acute encephalitis or a chronic paralytic disease (Bailey et al., 1949; Lampert et al., 1973; Le Provost et al., 1975; Hirano et al., 1980; Knobler et al., 1981). Intrathecal inoculation of JHM virus (JHMV) into rats has been shown to cause an acute necrotizing encephalomyelitis or a progressive demyelinating disease (Nagashima et al., 1978, 1979; Hirano et al., 1980; Sorensen et al., 1980, 1982).

CNS infection of mice may be caused by a number of murine coronaviruses which have tropisms for various cell types including glia and neurons (Virelizier et al., 1975; Fleury et al., 1980; Knobler et al., 1981a). In the rat overt CNS symptoms are associated exclusively with JHMV (Hirano et al., 1980; Sorensen et al., 1980). In rats JHMV can persist in the CNS for considerable periods without overt signs of disease (Nagashima et al., 1979; Sorensen et al., 1980). However, the mechanism of virus spread as well as the specific regions of the CNS and the cell types functioning as repositories there for the virus are yet to be identified. Nor is it clear whether JHMV persists as an infec-
tious entity at low titers or as a truly latent virus which may be triggered into replication.

Local production of antibodies in the CNS compartment is apparent in various neurologic diseases such as multiple sclerosis (MS) (Arnadottis et al., 1982), varicella zoster meningoencephalitis (Vartdal et al., 1982), Visna virus infection of sheep (Martin et al., 1982), and Semliki Forest virus-induced demyelination in mice (Parson and Webb, 1982). Although it is clear that the rat may exercise profound control over the outcome of the infectious process initiated by JHMV, since susceptibility to disease is both age related and genetically controlled (Sorensen et al., 1982), it remains to be established whether the onset of resistance is connected with the appearance of intracerebral immune responses. It is also uncertain whether such responses offer protection, in view of previous observations that neutralizing antibodies in the serum do not appear to influence the disease process (Sorensen et al., 1982). However, the presence of antibodies against OC43 and 229E, human coronaviruses serologically related to the murine strains, in the sera of a high proportion of the human population (Macnaughton, 1982) and intrathecal antibodies in some MS patients (Salmi et al., 1982; Hovanec and Flanagan, 1983) give the rat/coronavirus model particular relevance in attempts to elucidate the etiology of the human disease.

The present study was initiated to determine whether the spread of virus during acute paralytic and asymptomatic phases could be traced by antigenic and nucleic acid probes and whether the disease process is accompanied by a localized antibody response in the CNS. A preliminary account of some of this work was presented at the 1983 EMBO Workshop on Coronaviruses (Sorensen et al., 1984).

**MATERIALS AND METHODS**

**Viruses**

JHMV and MHV-3 were propagated and plaque assayed on L-2 mouse fibroblasts as previously described (Sorensen et al., 1980). Pooled virus lysates and uninfected L-2 cell inocula for intracerebral (ic) or intraperitoneal (ip) injections were prepared as previously described (Sorensen et al., 1980).

**Animals**

Wistar Lewis (WL) and Wistar Furth (WF) rats, originating from sources described previously (Sorensen et al., 1980), were bred in house. The ic inocula contained $5 \times 10^4$ to $1 \times 10^5$ PFU JHMV or $4 \times 10^4$ to $8 \times 10^4$ PFU MHV-3 per injection, and the ip inocula about $3 \times 10^5$ PFU per injection of either virus. All inoculations were performed on suckling rats between 2 and 10 days of age.

**Histology and Immunofluorescence**

Rats were anesthetized and killed by decapitation and the brain and spinal cords were quickly exposed, sectioned longitudinally, and one half was quick frozen for subsequent RNA extraction while the contralateral half was fixed in ethanol: acetic acid (3:1, v/v). Following fixation and dehydration the tissues were embedded in wax and sectioned at 6 μm. To correlate histopathology with the distribution of viral antigen, adjacent sections were stained with hematoxylin and eosin (HE) or were reacted with mouse anti-JHMV antibodies followed by fluorescein conjugated goat anti-mouse-IgG antibodies. These sections were then examined under bright field or uv fluorescence optics to determine the distribution of JHMV antigens and overt pathological lesions.

**Detection of JHMV-Specific Antibodies by Radioimmune Assay (RIA)**

Free virus was concentrated from supernatant fluids of infected L-2 cell monolayers by centrifugation then purified on a 15–45% sucrose gradient. IgG antibodies specifically against JHMV from hyperimmune rat anti-JHMV serum were purified and then coupled to cyanogen bromide-activated Sepharose 4-B. The virus-specific antibody was eluted as described by Lecomte and Tyrrell (1976), and the protein concentration determined accord
ing to Lowry (1951). JHMV-specific antibody of predetermined concentration was utilized throughout as the standard.

Affinity-purified anti-rat-IgG (Flow Laboratories) was tested for specificity by immunoelectrophoresis, then was radioiodinated with 125I (New England Nuclear Radiiodination Kit) employing the lactoperoxidase procedure of David and Reisfeld (1974). Free iodine was removed and the iodinated IgG was diluted to 10^6 cpm/μg protein.

To minimize contamination by blood of the cerebrospinal fluid (CSF), anesthetized rats were exsanguinated by cardiac puncture prior to making the cisternal tap, as described by Griffin (1981). All CSF samples were centrifuged and the resulting pellets examined for the presence of erythrocytes. Samples containing red blood cells were discarded.

As a direct test for the leakage of IgG from the serum to the CSF, radioiodinated rat IgG was injected intravenously into rats with hind-leg paralysis following JHMV infection and the CSF/serum ratio of 125I counts determined 18 hr later. No significant leakage of IgG into the CSF could be detected.

JHMV- or MHV-3-specific IgG antibodies in serum and CSF of rats were determined by an indirect solid-phase RIA procedure using flexible microtiter plates coated with 5 X 10^6 PFU JHMV per well then incubated with 100 μl 10% BSA in PBS/well followed by washing. Serum and CSF were diluted appropriately and 25-μl samples were delivered to each well and the plate was incubated at 37° for 2 hr, then washed. Finally each well received 25 μl (1 μg) radioiodinated goat anti-rat-IgG, incubated at room temperature for 60 min, washed, air dried, and assayed for 125I content. The IgG concentration, in micrograms per milliliter, was calculated from the disintegrations per minute measured in the sample.

Preparation of JHMV Complimentary DNA

(a) Isolation of template JHMV mRNA. Total RNA was extracted from L-2 monolayers 9 hr postinfection with JHMV using guanidine-HCl as described by Strohman et al. (1977). Poly(A)-tailed RNA was then isolated by oligo(dT)-cellulose column chromatography. The poly(A)-tailed RNA was applied to the nitrocellulose-bound uninfected L-2 cell DNA and incubated for 3 hr at 50° with constant agitation removing cellular RNAs by hybridization. L-2 cell DNA had been isolated by chloroform:phenol extraction of uninfected cells and bound onto nitrocellulose (Parnes et al., 1981) at a concentration of about 2 μg DNA/mm². The unbound JHMV RNA was decanted and precipitated overnight. This JHMV template RNA was pelleted, dissolved in 10 mM Tris–HCl, pH 7.4, and stored at −70°.

(b) Synthesis of 32P-labeled complementary DNA. Complementary DNA (cDNA) was synthesized and isolated as described by Coulter-Mackie et al. (1980) using the JHMV template RNA described above. The reaction mixture contained random calf thymus DNA primers, AMV reverse transcriptase (Life Sciences), [32P]dCTP (3200 Ci/mmol, New England Nuclear) and 0.1 mg/ml actinomycin D. The cDNA produced was suspended in 0.9 mg/ml uninfected L-2 cell DNA, allowed to anneal at 60° to a high Cot value (>500, about 20 hr) and the remaining ssDNA species were selected by hydroxyapatite column chromatography. The ssDNA-containing eluates, representing about 75% of the total 32P applied, were pooled, dialyzed against 3 mM EDTA, and precipitated. The cDNA precipitate was centrifuged at 16,000 g and resultant pellet was resuspended in 1.0 ml annealing buffer (50% v/v deionized formamide, 3X SSC, 1X Denhardt’s buffer (Denhardt, 1966), 1 mg/ml yeast RNA, 100 μg/ml alkaline-sheared single-stranded salmon sperm DNA, 0.01 M HEPES, pH 7.4) and stored at −20° until used for dot blotting. Both JHMV genomic and mRNA were detected by this cDNA in Northern analysis (Alwine et al., 1977; Coulter-Mackie et al., 1980) of JHMV-infected L-2 cell RNAs (data not shown).

Extraction of RNA from CNS Tissues

RNA extracts were made of the regions of the CNS defined in Fig. 1. These regions included the telencephalon, diencephalon/mesencephalon, cerebellum, pons/mye-
Telencephalon  Diencephalon  Cerebellum  Pons  Cervical Spinal Cord

Cerebrum  Rhombencephalon  Spinal Cord

FIG. 1. A schematic representation of the rat brain according to Zeman and Innes (1963). RNA extracted from these major segments as well as the lumbar spinal cord and the optic nerves was probed for the presence of JHMV RNA by dot-blot analysis.

Intracerebral Antibody Responses

Determination of anti-JHMV IgG concentration in the CSF and serum by the RIA technique revealed that during both acute and paralytic diseases a significant increase in the anti-JHMV IgG CSF/serum ratio occurred (Table 1). Such increases in the CSF/serum ratios imply local production of IgG in the CNS (Schliep and Felgenhauer, 1978; Fleming et al., 1983). Production of anti-JHMV antibody in the CNS was apparent only in those rats in which disease occurred, presumably contingent upon, and associated with, viral replication. Inoculated rats without clinical or histological indications of disease, including all those inoculated IC with MHV-3 and some with JHMV, had only slight increases in anti-JHMV IgG CSF/serum ratios (<10) when compared with ratios of <1 in control rats inoculated IP with JHMV (Table 1). The animals which developed a rapidly fatal acute encephalomyelitis were found to have the highest average CSF/serum ratio (>150) while somewhat lower ratios were found in paralyzed animals (Table 1). Elevated levels of anti-JHMV IgG in the CSF were observed only in rats which displayed clinical and/or histological evidence of viral pathology. Indeed, rats killed 7 days postinoculation (dpi) and prior to any clinical indications of disease, could be grouped according to their average CSF/serum levels (Table 1). Elevated CSF/serum ratios (>150) were found only in animals which, upon histological examination, displayed obvious lesions. These ratios were comparable to those evident in animals undergoing acute disease. Rats
### TABLE 1

**ANTI-JHMV IgG Concentrations in Cerebrospinal Fluid (CSF) and Serum of Rats after Virus Inoculation**

| Virus strain and inoculation route | Days postinoculation | Mean concentration of Anti-JHMV IgG (µg/ml) | Average CSF/serum ratio (×10^4) |
|-----------------------------------|----------------------|-------------------------------------------|---------------------------------|
| Controls JHMV ip no disease*     | 21                   | 1370.0 ± 383.8b                          | 0.3                             |
| MHV, ic no disease               | 7-21                 | 2225.7 ± 940.6                           | 3.2                             |
| JHMV ic no disease               | 7-40                 | 2384.3 ± 766.0                           | 7.1                             |
| JHMV ic acute disease            | <15                  | 3018.0 ± 1235.1                          | 197.0                           |
| JHMV ic paralytic disease        | >21                  | 1408.3 ± 588.7                           | 111.0                           |
| JHMV ic asymptomatic no histopathology | 7           | 4648.0 ± 433.3c                          | 6.6                             |
| JHMV ic asymptomatic histopathology | 7           | 6010.0 ± 180.0c                          | 179.5                           |

* Disease absent as judged by a lack of clinical and histological indications.

b Standard error of the mean.

c High serum antibody concentrations may be indicative of circulating maternal antibodies in these neonatal rats (Griffiths et al., 1982).

without histological evidence of infection had an average CSF/serum ratio equivalent to that noted in inoculated animals which failed to develop any disease (Table 1). A smaller group of rats, distinguished by intermediate CSF/serum ratios (data not shown), was also identified. This group may be comprised of animals in which demyelinating lesions would have eventually developed. The RIA method as used here detected those subclasses of IgG able to bind any JHMV antigen including those capable of neutralizing the virus. Thus the IgG concentrations determined by the RIA procedure probably overestimated the IgG antibodies capable of restricting virus spread through the CNS while detecting only one class of the total anti-JHMV antibody present.

**Histopathology and JHMV Antigen Distribution**

Surveys of CNS tissue by means of HE-stained sections confirmed data previously reported (Sorensen et al., 1980) showing that the type and distribution of lesions was related to the acuity of the disease, age at inoculation, and the incubation period. Examination of sections adjacent to those stained with HE for the presence of antigen, by means of indirect immunofluorescence, revealed that the distribution of viral proteins in the CNS differed from that of the lesions. A few cells near necrotic lesions were positive (Fig. 2), but cells containing JHMV antigen were more abundant in areas which appeared to be histologically normal (Fig. 3) and those characterized by lymphocytic infiltrates. Neurons of the cerebrum and cerebellum were more frequently infected than previously realized. Positive cerebral neurons (Fig. 3) were detected in some rats 21 dpi and even later, despite the fact that those lesions recognizable by HE staining were exclusively of the demyelinating type, suggesting oligodendrocyte involvement. JHMV antigen-positive cells were evident in both neurons and glia of the spinal cord although paralyzed rats with histological lesions confined to myelinated tracts of the rhombencephalon and spinal cord also displayed antigen in both neu-
rons and glia of the more rostral regions of the CNS. The absence of cells with JHMV antigen in necrotic areas indicates that virus infection of specific areas of the CNS precedes the formation of lesions.

Detection of JHMV RNA

The detection of antigen-positive cells in the CNS by immunofluorescent microscopy enabled us to pinpoint sites where JHMV proteins were being expressed but provided no direct evidence about viral latency. To address the question of latency, CNS tissues were surveyed for the presence of JHMV RNA with a cDNA probe. The CNS regions which were examined, described diagrammatically in Fig. 1, included the telencephalon, diencephalon/mesencephalon, cerebellum, pons/myelencephalon, cervical and lumbar segments of the spinal cord and, whenever possible, the optic nerves. Using $^{32}$P-labeled cDNA in the dot-blot procedure it was found that RNA extracted from uninoculated rats did not hybridize with the probe (Fig. 4D). In contrast, after infection, JHMV RNA became widely distributed in the CNS of animals displaying clinical and histological signs of disease. High concentrations of JHMV RNA were detected in extracts from the CNS of rats with a generalized encephalomyelitis (Fig. 4A). Viral RNA could be detected throughout the CNS as early as 6 dpi. The distribution of JHMV RNA was more extensive than that of histological lesions associated with the neurological disease process. Although both the pons/myelencephalon and cervical spinal cord contained viral RNA sequences at 6 dpi, the histological lesions were found to be concentrated in rostral regions of the CNS (Fig. 5).

Hybridization of the $[^{32}$P$c]$DNA probe with extracts from rats afflicted with hindleg paralysis, in which demelination is usually confined to the pons/myelencephalon, cerebellum, and/or spinal cord (Sorensen et al., 1980), revealed the presence of JHMV RNA in the pons/myelenceph-
FIG. 3. A sagittal section through the cerebrum of a 20-day-old rat inoculated ic at 10 days of age. Hematoxylin and eosin staining (A) illustrates an area of gray matter apparently free of pathology. However, the adjacent section (B), examined after immunofluorescent staining for JHMV antigens, contains several cells which are positive for viral antigen, some with the morphology of neurons. Bar = 0.03 mm.

alon and sometimes in the cerebellum (Fig. 4B). Viral RNA was present less frequently in the cervical spinal cord although histological lesions were readily apparent, and in the histologically normal diencephalon/mesencephalon regions (Figs. 4B, 5). The telencephalon region of paralyzed animals, which, likewise, was devoid of lesions (Fig. 5), occasionally contained JHMV RNA (Fig. 4B).

Rats not killed during the paralytic episode frequently regained normal coordination. RNA extracts from the CNS tissues of these animals were usually negative for the presence of JHMV RNA by dot-blot analysis (Fig. 4C). However, small amounts of viral RNA were detectable in the pons/myelencephalon and cervical spinal cord of one rat killed 7 days after recovering from hind-leg paralysis (Fig. 4C), whereas in another rat killed 2 weeks post recovery, JHMV RNA was not detectable (Fig. 4C). With two exceptions, animals without clinical symptoms or histological evidence of disease were negative for the presence of viral RNA (Fig. 4E). It is, nevertheless, significant that in this group two of the eight rats surveyed had detectable JHMV RNA although at low concentrations, in the telencephalon and diencephalon/mesencephalon when killed at 138 and 163 dpi (Fig. 4E). Thus prolonged, and presumably latent, infection of the rat CNS can occur despite an absence of disease symptoms.

Our previous histopathological surveys of rats inoculated with JHMV revealed that CNS lesions had a bilateral distribution in the CNS, although not necessarily at directly opposite sites (Sorensen et al., 1980). This finding indicated that a histological examination of one hemisphere and dot-blot probing for the presence of viral RNA in the contralateral hemisphere could provide comparable data. It is, therefore, significant that the current survey, while demonstrating a correlation between the presence of lesions in a particular region of the CNS and the occurrence of viral RNA, also revealed that
viral RNA was often present (Fig. 5) in the absence of histological lesions.

**DISCUSSION**

Our present study indicates that rats succumb to JHMV infection despite the capacity to mount an IgG response localized in the CNS. The concentrations of IgG in the CSF reported here are comparable to those determined in other viral infections of the CNS (Schliep and Felgenhauer, 1978; Martin et al., 1982). Indeed, the production of anti-JHMV IgG in the CNS correlated with an overt disease process since elevated CSF/serum ratios were recorded only in rats with clinical and/or histological evidence of disease. Despite the presence of virus-specific CSF IgG there was apparently no suppression of the continuing CNS infection. However, since the RIA method used polyvalent antibodies, IgG reactive with all JHMV antigens were detected, not exclusively...
those involved with virus neutralization. These data do not reveal the concentrations of JHMV-neutralizing antibodies in the CSF. In contrast, a survey of JHMV-neutralizing antibodies in the serum (Sorensen et al., 1982) indicated that after a single ic or ip injection the titers were not significantly greater than those of uninoculated controls. Thus it is possible that production of anti-JHMV antibody in the rat model does, in fact, occur exclusively or primarily in the CNS compartment.

Electron microscopic examination of rat CNS tissues indicated that during the prolonged demyelinating disease, JHMV infection appears to be confined to the glia (Sorensen et al., 1980). In the present analysis, however, viral antigen was frequently detected in cells with neuron morphology, although it has not been established whether the cells involved were producing infectious JHMV particles or only viral protein. These and previous observations (Sorensen et al., 1980) imply that JHMV can persist and replicate within neurons and oligodendrocytes without causing cell degeneration and death or eliciting an immune attack which might lead to tissue necrosis and suppression of the infection. In mice Knobler et al. (1981b) have similarly shown that neurons of SJL mice are a primary target for JHMV infection. Furthermore, as in the rat, SJL mice exhibit an age-dependent resistance to CNS infection following an ic challenge with JHMV. Specific tropisms of JHMV for primary CNS cultures enriched in oligodendrocytes and of MHV-3 for cultures of astrocytes (Sorensen et al., 1983; Beushausen and Dales, in preparation) have also been found. Thus our previous (Sorensen et al., 1980, 1982) and current observations indicate that both glial and neuronal cells can serve as primary targets and repositories for coronaviruses within the CNS.

A continuing spread of JHMV throughout the CNS, despite the development of a localized antibody response in the CSF, suggests that demyelination occurs as a consequence of oligodendrocyte infection. However, the reported association, in the rat, between JHMV infection of the CNS and proliferation of cytotoxic T cells reactive against myelin basic protein (Watanabe et al., 1983), implies that demyelination could occur as a consequence of autoimmune phenomena analogous to those involved with experimental allergic encephalomyelitis (Paterson and Day, 1982).

The presence of cells containing viral antigen in CNS regions with normal histological appearance, either in the vicinity of or unrelated to lesions, correlates with previous electron microscopic identification of virus structures in oligodendrocytes distributed within normal tissue adjacent to lesions (Sorensen et al., 1980). These
data also correlate with detection of JHMV RNA in samples of CNS tissue taken from asymptomatic rats 4-5 months after inoculation. Furthermore, our current finding that specific CNS regions may be positive for JHMV RNA while devoid of histopathology, supports this interpretation. One should, nevertheless, be aware that an apparent absence of lesions could, at least in part, be ascribed to the limitations imposed by the size and numbers of histological samples that can be examined.

During the rapidly fatal, acute disease JHMV RNA may occur throughout the CNS. Despite the usual absence of histological lesions in the cervical spinal cord of acutely diseased animals (Sorensen et al., 1980) the presence of JHMV RNA in this tissue again implies that viral replication precedes the development of lesions. When the disease process resulted in demyelination, the distribution of viral RNA within the CNS was more restricted, so that the more rostral tissues had either little or no detectable JHMV RNA while in the rhombencephalon and, to a lesser extent, the cervical spinal cord, viral RNA was readily detectable.

Attempts to isolate infectious virus more than 6 dpi, from rats without clinical or histological indications of disease, have been unsuccessful (unpublished data) but maintenance of JHMV in the CNS of asymptomatic rats could be demonstrated following immunosuppression with cyclophosphamide (Sorensen et al., 1982). Thus, it seems likely that the viral RNA and antigen, which may linger in the absence of disease symptoms, are associated with the production of infectious particles below levels detectable by the infectivity assays usually employed.

In future explorations of the rat model it should be possible to determine the unique tropism for neural cell types of the various coronaviruses which have or lack the potential for causing neurologic disease (Hirano et al., 1980; Sorensen et al., 1983). Such studies, when coupled with investigations of primary brain cultures, may elucidate the mechanisms involved in maintenance of JHMV persistence or latency within the CNS.

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