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In Vivo and In Vitro Models of Demyelinating Disease: JHM Virus in the Rat Central Nervous System Localized by In Situ cDNA Hybridization and Immunofluorescent Microscopy

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In situ probing of central nervous system (CNS) tissues has made it possible to associate the presence of JHM virus (JHMV) RNA with individual cells in the rat CNS. The presence of viral RNA was not always associated with antigen expression. The in situ hybridization revealed that cerebellar Purkinje cells and hippocampal neurons were highly susceptible to JHMV infection during either acute or paralytic disease. In the paralytic disease, Purkinje cell neurons frequently contained viral RNA. This observation suggests that these neurons, and perhaps others, may be repositories for JHMV in rats that undergo prolonged infections.

The injection of mouse hepatitis virus strain JHM (JHMV) into the rat central nervous system (CNS) may cause either an acute encephalomyelitis or a paralytic demyelinating disease (6, 7, 11). The location of the histological lesions, as well as the extent of demyelination, can be correlated with the strain and age of the rat at the time of inoculation and the time elapsing before the initial clinical symptoms are noted (11). Our previous studies on the RNA extracted from CNS tissue with cDNA probes specific for the JHMV genome and mRNA revealed that viral infections initiated by intracerebral inoculation are quickly disseminated throughout the CNS (10). Although these dot blot assays of RNA extracts adequately localize viral RNA to specific regions of the rat CNS, they cannot be used to identify which cells are actually involved in the infection. Precise localization of viral RNA may, however, be achieved by means of in situ hybridization with suitable probes (1). Therefore, as an extension of our previous investigations of neurologic disease in the coronavirus-rat model, the dual detection, at the cellular level, of JHMV RNA and antigen(s) was undertaken.

MATERIALS AND METHODS

Wistar Furth rats, 5 to 10 days of age, were inoculated intracerebrally with about 5×10^4 PFU of JHMV per animal, as previously described (11). At necropsy, CNS tissue was quickly removed, fixed in ethanol-acetic acid (3:1 [vol/vol]), and subsequently dehydrated in graded ethanol solutions, embedded in paraffin, and sectioned at 10 μm. Tissue sections were floated on 0.1% Lepage white glue (13) and collected on glass slides which had been coated with Denhardt medium and acetylated (2). The sections were deparaffinized, rehydrated, acetylated (3), digested with pronase (0.5 μg/ml in 0.02 M Tris hydrochloride [pH 7.4]–0.002 M $CaCl_2$), washed, dehydrated, and air dried. Sections were exposed to the cDNA probe under a siliconized cover slip at room temperature for 48 h to allow hybrid formation.

The cDNA probe was synthesized with [^{35}S]-deoxycytidine 5′-[α-thio] triphosphate as substrate on a template of JHMV-specific poly(A)-tailed RNA by avian myeloblastosis virus reverse transcriptase, as described previously (10). Nonspecifically bound cDNA was removed by successive washes at room temperature with 50% formamide (0.01 M Tris hydrochloride [pH 7.5], 0.001 M EDTA, 0.6 M NaCl) and 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Sections were then dehydrated by graded ethanol solutions containing 0.3 M ammonium acetate, air dried, coated with Kodak NTB-2 nuclear track emulsion, stored for 48 to 96 h at 4°C, developed with Kodak D19, and stained with Harris hemotoxylin and eosin by the method of Humason (4). Hybridized tissue sections were routinely examined under bright-field and dark-field illumination, while JHMV antigen(s) were identified in adjacent sections by means of indirect immunofluorescent microscopy with polyclonal mouse anti-JHMV antiserum, as previously described (10).

RESULTS

CNS tissues from rats afflicted by either acute or paralytic JHMV infections were analyzed for the presence of viral RNA and antigen. These observations are described in detail below and are summarized in Table 1.

Acute disease. Histological examination of rats undergoing the rapidly developing acute disease showed that the initial identifiable lesions were concentrated in the telencephalon. As the incubation period lengthened from 6 to 14 days postinoculation, the lesions tended to be located in more caudal portions of the CNS and involved both neurons and glia. In situ hybridization of JHMV-specific probes with CNS tissues from rats early in the acute infection revealed the presence of JHMV RNA in some neurons of the hippocampus, particularly neurons of the stratum pyramidale (Fig. 1), although viral antigen and identifiable lesions did not appear until later.

Within this short period after infection, in situ hybridization could also localize virus-specific RNA to glia in the myelinated tracts of the cervical spinal cord (Fig. 2). Occasionally cells of the spinal cord grey matter also contained virus RNA (Table 1). Hippocampal neurons were essentially free of virus antigen during the early acute disease process while JHMV antigen was present in other neurons of the telecephalon of the same animals. With longer incubation

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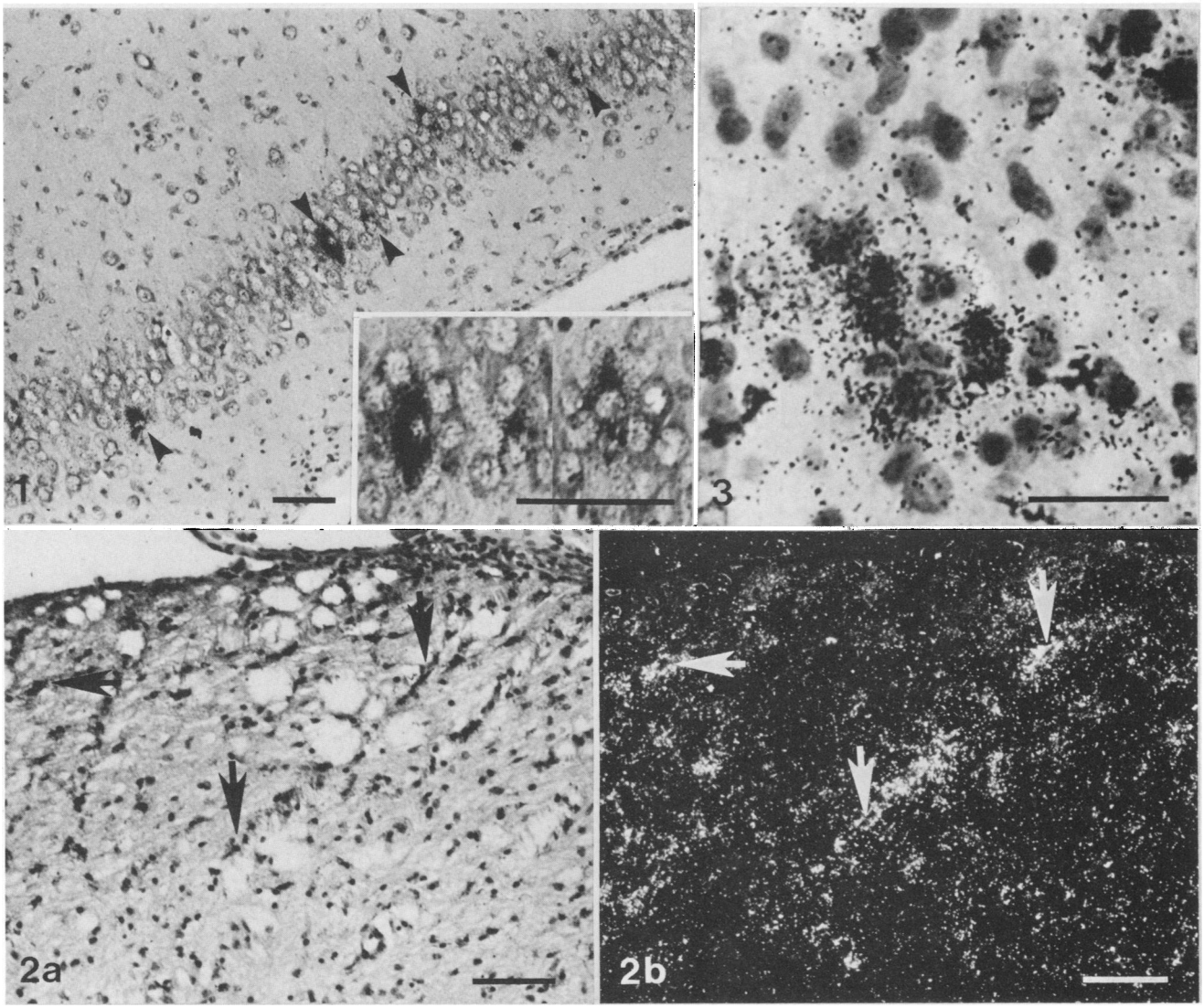

FIG. 1. In situ hybridization with JHMV-specific [^{35}S]cDNA followed by hemotoxylin and eosin staining of a selected area of the hippocampus of a rat suffering an acute JHMV infection. Note the accumulation of grains over several neurons (arrowheads). At higher magnification (insert), it can be seen that the grains occur predominantly over the cytoplasm of the neurons. Bar represents 30 μm.

FIG. 2. Longitudinal section of spinal cord from a rat suffering an acute JHMV infection, prepared as in Fig. 1, as seen under bright-field illumination (a) and as seen by dark-field illumination (b) to emphasize the silver grains. Arrows indicate identical cells in panels a and b. Bar represents 30 μm.

FIG. 3. An area of the myelencephalon of a paralyzed rat, prepared as in Fig. 1. A number of cells positive for JHMV RNA can be distinguished by the presence of overlying grains. Bar represents 10 μm.

periods, JHMV antigen could be identified in hippocampal neurons of acutely affected rats. The spinal cord of rats was generally devoid of antigen and without evidence of histopathology during the acute infection (Table 1).

These findings emphasize that the presence of virus-specific RNA could be demonstrated, by means of in situ hybridization with cDNA probes, in CNS tissues which were histologically normal and essentially negative for JHMV antigen(s).

Paralytic disease. Histological examination of CNS tissues from paralyzed rats showed that identifiable lesions were essentially confined to myelencephalon, cerebellum, and spinal cord. Occasionally, lesions could also be identified in the mesencephalon and the molecular and granule layers of the cerebellum. JHMV-specific probing of CNS tissues from paralyzed rats showed that some neurons of the telencephalon were occasionally positive for JHMV RNA (Table 1) without involvement of glia in this area.

During the paralytic disease viral RNA was frequently present within the myelencephalon (Fig. 3) and, more frequently, in Purkinje cell perikaryons (Fig. 4). However, adjacent granule cells (Fig. 4) and glia of the cerebellar white matter were rarely JHMV RNA positive (Fig. 5). Examination of spinal cord samples taken from rats with paralysis showed that JHMV RNA was present frequently in the glia of the white matter but also occasionally in neuronal cells of the grey matter (Table 1). The detection of viral antigen(s) in spinal cord tissues taken from paralyzed rats indicates that antigen expression occurred primarily in the myelinated tracts (Fig. 6). Thus, a close correlation was evident between

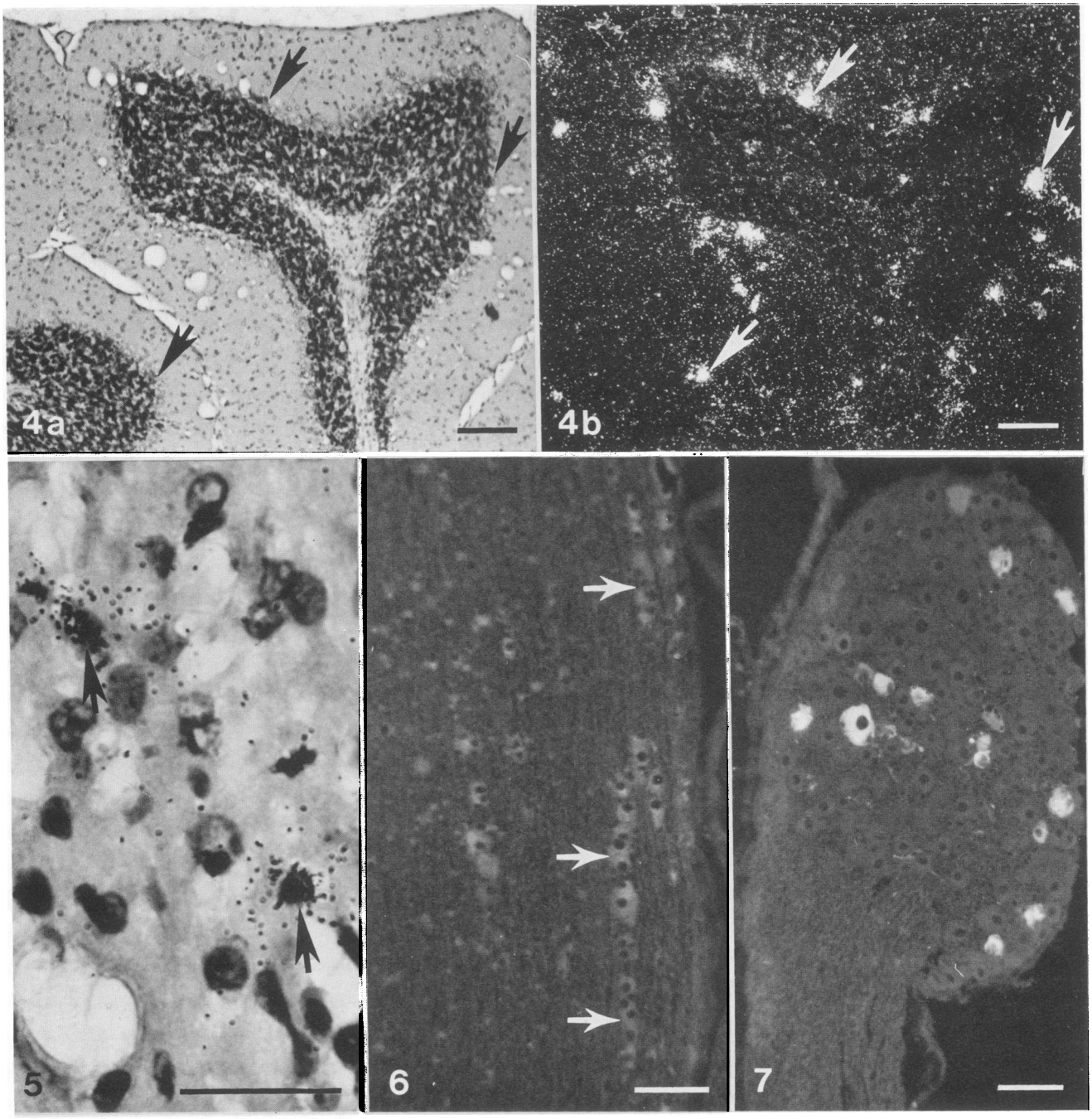

FIG. 4. A portion of the cerebellum of a paralyzed rat probed in situ with virus-specific [^{35}S]cDNA and stained with hemotoxylin and eosin, as seen under bright-field illumination (a) and as seen in dark-field illumination (b) to emphasize the silver grains. Note that the tissue appears to be histologically normal although clusters of silver grains, emphasized in panel b by dark-field illumination, can be identified over the Purkinje cell layer. Arrows point out identical cells in panels a and b. Bar represents 50 μm.

FIG. 5. An area of the cerebellar white matter of a paralyzed rat, prepared as in Fig. 4, illustrates the presence of glial cells positive for JHMV RNA (arrows). Bar represents 10 μm.

FIG. 6. Longitudinal section of spinal cord from a rat with progressive disease, examined under UV optics after staining for immunofluorescent microscopy. Note the presence of JHMV antigen-positive cells (arrows) between myelinated axons in an area where no demyelination is evident. Bar represents 30 μm.

FIG. 7. Longitudinal section of a spinal ganglion, prepared as in Fig. 6, demonstrates the presence of viral antigen in the perikaryons of several neurons in a rat 15 days postinoculation. Bar represents 30 μm.

the distribution of viral RNA and antigen(s) in the spinal cord (Fig. 6; Table 1). Such correlation, however, was not evident in Purkinje cell neurons of the cerebellum, where the presence of JHMV RNA was not invariably matched by the presence of detectable JHMV antigen(s) in adjacent sections. These findings emphasize once more the sensitivity and specificity of our cDNA when used for in situ probing of tissue sections.

TABLE 1. Distribution of viral antigen and RNA in the CNS of JHMV infected rats

CNS tissue	Distribution of viral antigen and RNA in rats with[a]:			
	Acute disease[b]		Paralytic disease[c]	
	Antigen	RNA	Antigen	RNA
Telencephalon	N	N, G	—	N[d]
Hippocampus	N[e]	N	N[d]	N[d]
Diencephalon and mesencephalon	G	N, G	—	G[d]
Cerebellum				
Molecular layer	—	—	G	G
Purkinje cells	—	N	N[d]	N
Granule layer	N	N	—	N[d]
Arbor vitae	G	—	—	GN[d]
Pons and myelencephalon	G	N, G	—	G
Spinal cord				
Grey matter	—	N[d]	—	N[d]
White matter	G[e]	G	G	G
Meninges	—	—	—	—
Ependyma	—	—	—	—
Choroid plexus	—	—	—	—
Perivascular cuffs	—	—	—	—
Endothelial lining	—	—	—	—

[a] Viral antigen was identified by indirect immunofluorescences with polyclonal mouse anti-JHMV antibodies. Viral RNA was detected by in situ hybridization with JHMV-specific cDNA. Cell types: N, neurons; G, glia (exact identification not made). —, Not found.
[b] Rats which died or were killed in extremis 1 to 15 days postinoculation.
[c] Rats which displayed hind leg paralysis at death.
[d] Noted only rarely.
[e] Negative early in acute disease but positive at later times.

A novel observation was the detection of JHMV antigen(s) in neurons of the spinal ganglia from a rat undergoing an acute infection (Fig. 7). It remains to be determined whether these cells of the peripheral nervous system (PNS) support the production of infectious virions and, if so, whether any of these progeny can be disseminated to neurons or Schwann cells elsewhere in the PNS. To date there is no histological evidence that coronaviruses cause demyelination in the PNS of rats.

During either the acute or the demyelinating disease in the JHMV-infected rat, endothelial lining of CNS blood vessels, perivascular cuff lymphocytes, and cells of the meninges, ependyma, and choroid plexus were consistently free from viral antigen and RNA (Table 1), indicating that these tissues do not support replication of this neurotropic agent.

DISCUSSION

Previous analyses of acutely diseased rats, in which the distribution of viral antigen was determined by immunofluorescent microscopy, showed that JHMV infection can occur in both neurons and glia (6, 10). In particular, the infection involved the neurons of the hippocampus and the Purkinje cells of the cerebellum (6). The same technique showed that the human coronavirus, OC43, infects cerebellar Purkinje cells in the mouse (8). In infections with reovirus type 3 (Dearing strain) and its less virulent hemagglutinin variants, a similar predilection for neurons of the murine hippocampus was also found (12). The present study shows that during an acute JHMV infection, neurons of the telencephalon become an early target and express viral antigen. Hippocampal neurons, although containing viral RNA early in the acute infection, do not express significant amounts of antigen until days later when they and the Purkinje cells of the cerebellum become antigen positive.

An unexpected finding in this study was the infection of PNS neurons in rat spinal ganglia by JHMV during an acute infection. The dissemination of JHMV to the ganglia did not appear to be accompanied by pathological changes in the PNS. Similar observations have been reported during OC43 virus infection of mice (8), and murine dorsal root neurons have been shown to produce infectious particles in vitro (9). It has yet to be determined whether ganglionic neurons of the rat can produce infectious JHMV. The absence of PNS demyelination, noted in the present and previous studies (6, 7; O. Sorensen, unpublished data), correlates with the inability of JHMV to infect rat Schwann cells in primary culture (S. Beushausen and S. Dales, unpublished observations) and of OC43 virus to infect explanted murine Schwann cells (9). By contrast, JHMV readily establishes a persistent infection in the rat Schwannoma cell line, RN2-2 (5).

In situ hybridization with ^{35}S-labeled cDNA has proved to be a sensitive tool for precise detection of JHMV RNA during the paralytic disease in the rat CNS. In some instances, the probe has revealed the presence of viral RNA in the absence of detectable antigen. It should, however, be emphasized that our failure to detect virus antigen at the cellular level is not free from ambiguity, since cDNA probing and immunofluorescent microscopy were carried out independently on serial tissue sections. During the paralytic disease, and in asymptomatic rats, the continued presence of viral RNA in Purkinje neurons of the cerebellum and perhaps in other neuron types (10) implies that these cells may function as virus repositories, a suggestion stemming also from our previous dot blot analysis of RNA extracts from specified regions of the rat CNS (10). We anticipate that in future applications of the in situ hybridization technique it will be possible to define the cell types in the rat CNS which may harbor JHMV throughout the prolonged asymptomatic phase of infection, thereby providing a virus reservoir which occasionally induces a delayed paralytic disease (11).

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