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Characterization of Murine Hepatitis Virus (JHM) RNA from Rats with Experimental Encephalomyelitis

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When Wistar Furth rats are inoculated intracerebrally with the murine hepatitis virus JHM they often develop a demyelinating disease with resulting hind leg paralysis. Using an RNA transfer procedure and hybridization kinetic analysis, the virus-specific RNA in these rats was characterized. The pattern of JHM-specific RNA varied with individual infections of Wistar Furth rats. However, two species of JHM-specific RNA, the nucleocapsid and a 2.1-2.4 $\times$ 10^6-Da RNA species were generally present. A general decrease in JHM-specific RNA in brains and spinal cord samples taken later than 20 days postinoculation was observed; however, JHM-specific RNA persisted in the spinal cord longer than in the brain of these rats.

INTRODUCTION

Murine hepatitis virus JHM can cause a demyelinating disease when injected intracerebrally (ic) into rats (Nagashima *et al.*, 1978; Sorensen *et al.*, 1980). Specifically, Wistar Furth rats, injected ic at 10 days of age, generally developed hind leg paralysis or paresis with white matter involvement (Sorensen *et al.*, 1980). There are many similarities between Wistar Furth rats with JHM-induced hind leg paralysis and MS in humans. Both diseases are characterized by a demyelination with destruction of glial cells and a relative sparing of axons (see review by Andrews, 1972; Oppenheimer, 1976; Sorensen *et al.*, 1980). In addition, JHM infection of the CNS of rats and MS in humans is characterized by periods of active demyelination followed by remission (Andrews, 1972; Rose, 1972; Sorensen *et al.*, 1980). Some degree of remyelination can be observed in MS patients (Andrews, 1972). The suggestion of remyelination has also been observed in JHM-infected rats (Sorensen *et al.*, 1980). In addition, the distribution of the demyelinating lesions is similar in JHM-infected Wistar Furth rats and in MS. In both cases the cerebrospinal axis, including the optic nerve, is involved (Oppenheimer, 1976; Sorensen *et al.*, 1980).

The clinical disease induced by JHM inoculation of Wistar Furth rats and its resulting lesions have been described (Sorensen *et al.*, 1980; Nagashima *et al.*, 1978). However, the nature of the viral RNA produced during these infections is still unknown. Therefore, the purpose of this study was to extend clinical and pathological findings by examining the virus-specific RNA sequences in JHM-infected Wistar Furth rats using RNA transfer techniques and hybridization kinetics.

MATERIALS AND METHODS

Cells and virus. The JHM strain of mouse hepatitis virus was obtained from the American Type Culture Collection (Rockville, Md). The viral inocula were propagated on L-2 murine fibroblast cells (Rothfels *et al.*, 1959). Five- or ten-day-old rats received approximately 5 $\times$ 10^4 PFU of JHM in a 20-μl intracerebral (ic) inoculation.

Preparation of tissue. Rat pups were killed by decapitation. The calvarium was removed and the brain was exposed. The

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vertebral column was sectioned longitudinally and the spinal cord was removed. The following samples were processed for microscopic examination: sagittal and coronal sections of cerebrum, sagittal sections of mesencephalon, and representative sagittal sections of the cervical and lumbar regions of the spinal cord. All histopathology samples were fixed overnight by immersion in 10% neutral buffered formalin. The tissues were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. The remainder of the tissue, used to isolate infected-cell RNA, was quick frozen by immersion in liquid nitrogen and stored at −70°.

Isolation of RNA. Frozen samples were placed in a cold solution of 7.6 *M* guanidine-HCl: 2.0 *M* KAc (19:1, v/v), and 1% antifoam C. The tissue was quickly dispersed with a Virtis homogenizer and glass dounced on ice. The procedure of Strohman *et al.* (1977) as modified by Dr. G. Mackie (Department of Biochemistry, University of Western Ontario) was used to isolate the cellular and viral RNA (see Cheley *et al.*, 1981a for details of procedure). In addition, cellular and viral RNA was isolated by the same procedure from uninfected and JHM-infected L-2 cell monolayers.

Preparation of complementary DNA. Murine hepatitis virus-specific ^{32}P-labeled complementary DNA([^{32}P]cDNA), sp act of 2-4 × 10^8 cpm/μg, was prepared from infected-cell RNA as described by Cheley *et al.* (1981a). Before use [^{32}P]cDNA was annealed with uninfected L-2 cell RNA and virus-specific sequences were selected on an hydroxylapatite column (Cheley *et al.*, 1981a). The selected probe did not anneal with uninfected cell RNA (Fig. 1).

RNA transfer and hybridization kinetic analysis. The RNA samples were denatured with glyoxal, subjected to electrophoresis in 1.5% agarose gels, and transferred to diazobenzyloxymethyl (DBM) paper. The RNA was then hybridized with virus-specific [^{32}P]cDNA (McMaster and Carmichael, 1977; Alwine *et al.*, 1977) and the JHM virus-specific RNA species were

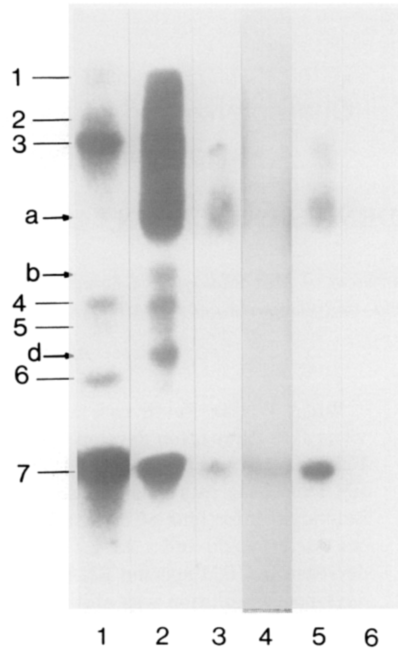

FIG. 1. RNA transfer analysis of RNA extracted from JHM virus-infected Wistar Furth CNS tissue or from JHM-infected L-2 cells. RNA was extracted either from brain or spinal cord samples from Wistar Furth rats that were inoculated intracerebrally 10 days after birth with JHM virus or from JHM-infected tissue culture (L-2) cells. The RNA was subjected to electrophoresis in a 1.5% agarose gel (40 V, 18 hr). The RNA was then transferred to DBM paper and annealed to a murine hepatitis virus-specific ^{32}P-labeled cDNA. Virus specific RNA was visualized by autoradiography. (1) RNA (30 μg) from JHM-infected L-2 cells. (2) RNA (24 μg) extracted from the spinal cord of Wistar Furth rat 10JWF5. This animal was sacrificed 15 days postinoculation (pi). (3) RNA (22.4 μg) extracted from the spinal cord of 10JWF11 (sacrificed 17 days pi). (4) RNA (22.5 μg) extracted from the brain of 10JWF12 (sacrificed 18 days pi). (5) RNA (22.1 μg) extracted from the spinal cord of 10JWF12. (6) RNA (30 μg) from uninfected L-2 cells. On the vertical axis the numbers and lines designated JHM-specific RNA species that were present in JHM-infected tissue culture (L-2) cells (Lai *et al.*, 1981; Spaan *et al.*, 1981; Leibowitz *et al.*, 1981; Cheley *et al.*, 1981a, b). The letters and arrows designate JHM-specific RNA species that were not present as major viral species in JHM-infected L-2 cells (Lai *et al.*, 1981; Spaan *et al.*, 1981; Leibowitz *et al.*, 1981; Cheley *et al.*, 1981a, b). RNA species "a" is the 2.1-2.4 × 10^6-Da species referred to in the text.

visualized by autoradiography (Swanstrom and Shank, 1978).

The hybridization kinetic analysis was performed as previously described (Cheley *et al.*, 1981a).

RESULTS

Murine Hepatitis Virus (JHM) RNA Species in Intracerebrally Inoculated Rats

Wistar Furth rats were inoculated ic at 5 or 10 days of age and generally developed hind leg paralysis or paresis (Table 1). Lesions seen in the brain and spinal cord were similar to those previously described (Sorensen *et al.*, 1980). In those rats which survived for longer than 3 weeks postinoculation (pi), lesions were often more extensive in the white matter including optic nerve, cerebrum, mesencephalon, metencephalon, and spinal cord (Table 1). Changes in the white matter were characterized by vacuolation, hypertrophy, and hyperplasia of astroglial cells, and minimal to moderate mononuclear cell infiltration. Lesions were focal to segmental in nature. Hypertrophy of endothelial cells and perivascular cuffing with mononuclear cells were common findings. In severely affected areas, there was partial to complete obliteration of the normal architecture by the destructive inflammatory process. Gray matter lesions were characterized by destruction of neurons and astroglial cells, proliferation of endothelial cells lining capillaries, and mononuclear and polymorphonuclear cell infiltrations.

Using RNA transfer analysis, we examined the JHM-specific RNA sequences in these JHM-inoculated Wistar Furth rats. Our data revealed heterogeneity in the JHM-specific RNA species that were present in individual rats. Some RNA samples from inoculated rats had relatively few virus-specific RNA species (Fig. 1, lanes 3-5). However, all 10 examined spinal cord and brain samples from 5- and 10-day-old inoculated rats contained the 0.8×10^6 Da JHM nucleocapsid mRNA species; in addition, all five spinal cord and four out of five brain samples contained a major RNA species of approximately $2.1\text{-}2.4 \times 10^6$ Da (RNA species "a" in Figs. 1 and 2). This $2.1\text{-}2.4 \times 10^6$-Da species is of particular interest since it is not visible as a major viral RNA species in JHM-infected L-2 cells but was present as a dominant, somewhat heterogeneous JHM RNA species in the inoculated rats (Figs. 1 and 2). This RNA species did not appear to be a nonspecific breakdown product of higher-molecular-weight RNA since it was reproducibly present as a major species of viral RNA even though ethidium bromide staining indicated that the 18 and 28 S RNA were present as undegraded, discrete species (Fig. 3). In addition the $2.1\text{-}2.4 \times 10^6$-Da species was present whether or not the JHM-specific 3×10^6 Da and genomic RNAs were present as major discrete species (Figs. 1 and 2). In addition, many brain and spinal cord samples from inoculated rats also contained other viral-specific RNA species which were not detected as major viral RNA species in JHM-infected L-2 cells (Figs. 1 and 2).

Detectability of Total JHM-Specific RNA

Using the RNA transfer procedure we were able to detect JHM-specific sequences in all the brain and spinal cord samples from rats killed at 11-19 days postinoculation (pi) (Figs. 1 and 2; Table 2). When animals were killed at 22 days or longer pi, JHM-specific RNA was no longer detectable using standard RNA transfer procedures and a cDNA with a sp act of $2\text{-}4 \times 10^8$ cpm/μg. (Fig. 2; Table 2). The inability to detect viral RNA in these samples was not due to degradation of the RNA since the cellular 18 and 28 S RNAs were still present in these samples as discrete RNA species after agarose gel electrophoresis (Fig. 3). These results were confirmed and expanded by hybridization kinetic analysis. JHM-specific RNA was not detectable by hybridization kinetics in the brains of animals injected ic at 10 days of age and sacrificed at 24 or 36 days

TABLE 1

EXPERIMENTAL JHM ENCEPHALOMYELITIS: INCIDENCE OF LESIONS IN GRAY AND WHITE MATTER

Animal number	Days post-inoculation	Optic nerve WM[a]	Cerebrum		Mesencephalon		Metencephalon		Spinal cord		Symptoms at time rat sacrificed
			WM	GM[b]	WM	GM	WM	GM	WM	GM	
10JWF5[c]	15	NE[d]	NE	NE	NE	NE	NE	NE	NE	NE	Hind leg paresis
10JWF11	17	NE	++	+	+	+	−	−	+	+	Hind leg paralysis
10JWF12	18	+++	+	+	+	+	+	+	−	+	Hind leg paresis
10JWF14	24	+++	−	−	+	+	−	−	+	+	Hind leg paralysis
10JWF15	24	+++	+	−	−	−	+	+	++	−	Hind leg paresis
10JWF16	25	++	++	−	++	+	++	−	+	+	Stunted growth Hind leg paralysis
10JWF17	28	+	−	−	++	+	−	−	+	+	Hind leg paralysis
10JWF19	36	+	−	−	−	−	+	−	++	−	Recovered from Hind leg paresis
5JWF2	10	NE	++	++	++	++	−	−	++	++	Hind leg paresis
5JWF11	11	+	+	++	−	+	−	+	++	+	Hind leg paralysis
5JWF12	25	NE	−	−	++	−	+++	−	+++	+	Stunted growth Hind leg paralysis
5JWF13	33	−	−	−	−	−	−	−	−	−	Recovered from Hind leg paresis

[a] White matter lesions.
[b] Gray matter lesions.
[c] First number refers to the age of the Wistar Furth rat at ic inoculation. Thus 10 JWF5 was inoculated at 10 days of age, and 5JWF2 was inoculated at 5 days of age.
[d] NE = Not examined; − = no lesions; + = minimal lesions; ++ = moderate lesions; +++ = marked lesions.

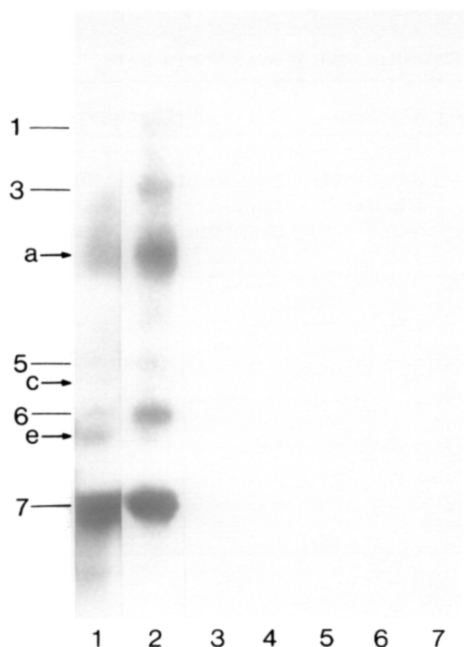

FIG. 2. RNA transfer analysis of RNA extracted from JHM virus-infected Wistar Furth CNS tissue. RNA was extracted from brain and spinal cord tissue from Wistar Furth rats that were littermates and were inoculated intracerebrally 5 days after birth with JHM virus. The RNA was subjected to electrophoresis in a 1.5% agarose gel (40 V, 18 hr). The RNA was then transferred to DBM paper and annealed to a murine hepatitis virus-specific ^{32}P-labeled cDNA. RNA was visualized by autoradiography (1) RNA (20 μg) extracted from the brain of Wistar Furth rat 5JWF11. The rat was sacrificed at 11 days postinoculation (pi). (2) RNA (22.1 μg) extracted from the spinal cord of 5JWF11. (3) RNA (20.6 μg) extracted from the brain of 5JWF12 (sacrificed at 25 days pi). (4) RNA (21.4 μg) extracted from the spinal cord of 5JWF12. (5) RNA (20 μg) extracted from the brain of 5JWF13 (sacrificed at 33 days pi). (6) RNA (21.3 μg) extracted from the spinal cord of 5JWF13. (7) RNA (20.9 μg) extracted from the brain of an uninfected littermate 5JWF16. On the vertical axis the number and lines designate JHM-specific RNA species that are present in JHM-infected tissue culture (L-2) cells (Lai *et al.*, 1981; Spaan *et al.* 1981; Leibowitz *et al.*, 1981; Cheley *et al.*, 1981a, b). The letters and arrows designate JHM-specific RNA species that are not present as major viral species in JHM-infected L-2 cells (Lai *et al.*, 1981; Spaan *et al.*, 1981; Leibowitz *et al.*, 1981; Cheley *et al.*, 1981a, b). RNA species "a" is the 2.1-2.4 $\times$ 10^6-Da species referred to in the text.

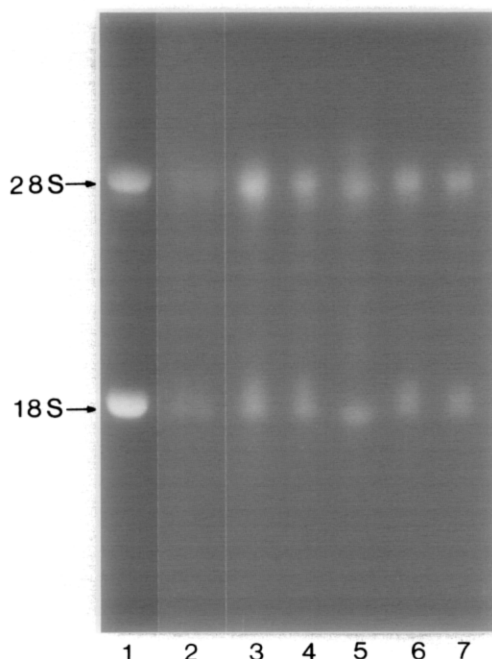

FIG. 3. Presence of discrete cellular 18 and 28 S RNA species indicated RNA samples from Fig. 2 were not degraded. The samples described in the legend to Fig. 2 were stained with ethidium bromide before the RNA species in the agarose gel were transferred to DBM paper (Northern Transfer); the cellular 18 and 28 S ribosomal RNA species were then visualized by illuminating the gel with uv light. (1) RNA from the brain of 5JWF11. (2) RNA from the spinal cord of 5JWF11. (3) RNA from the brain of 5JWF12. (4) RNA from the spinal cord of 5JWF12. (5) RNA from the brain of 5JWF13. (6) RNA from the spinal cord of 5JWF13. (7) RNA from the brain of an uninfected littermate 5JWF16.

pi, but JHM RNA sequences were detected by hybridization kinetics in the spinal cords of animals sacrificed at 25 or 28 days pi (Tables 2 and 3). Brain and spinal cord lesions were observed in all of these 10-day-old inoculated rats. Thus the brain lesions can persist after detectable viral infection has ceased (Tables 1 and 3). In contrast to the 10-day-old inoculated rats, we could detect (using hybridization kinetics) JHM-specific RNA in the brain of one rat (5JWF12) that was inoculated ic at 5 days of age and killed at 25 days pi.

TABLE 2

DETECTABILITY OF JHM RNA IN INTRACEREBRALLY INOCULATED WISTAR FURTH RATS

Age at inoculation (days)	Range of days postinoculation rats sacrificed	Brain (B) or spinal cord (c) sample	RNA transfer analysis: Number of samples with detectable RNA / Number of samples tested	Crt[a] analysis: Number of samples with detectable RNA / Number of samples tested
10	15–18	B	2/2	1/1
		C	3/3	1/1
10	22–36	B	0/6	0/3
		C	0/3	2/2
5	11–19	B	4/4	2/2
		C	3/3	1/1
5	25–33	B	0/2	1/1[b]
		C	0/2	NT[c]

[a] Product of RNA concentration and time (Birnstiel *et al.*, 1972).
[b] Animal with focal leucoencephalitis.
[c] Not tested.

TABLE 3

QUANTIFICATION OF JHM-SPECIFIC RNA IN INTRACEREBRALLY INOCULATED WISTAR FURTH RATS

Animal[a]	Time post-inoculation (days)	% Annealing	Crt[b] ($\times 10^{-4}$)	% of JHM-specific RNA compared with JHM-infected$_3$ L-2 cells ($\times 10^3$)
10JWF14B	24	8	2.2	0
10JWF15B	24	14	2.1	0
10JWF19B	36	7	3.3	0
10JWF17C	28	72	2.9	0.5[c]
10JWF16C	25	62	1.8	0.6
5JWF12B	25	73	1.9	0.8
5JWF8B	12	73	2.0	0.9
		63	1.0	
5JWF9B	12	100	1.9	1.0
		17	0.8	
5JWF9C	12	84	0.8	3.0
Uninfected L-2 cell	—	10	1.8	—
		6	0.9	
		5	0.7	

[a] The first number designates the age of the rat at inoculation. B designates a brain sample and C a spinal cord sample. Thus 10JWF14B is a brain sample from a Wistar Furth rat that was inoculated intracerebrally with JHM when it was 10 days old.
[b] Product of RNA concentration and time (Birnstiel *et al.*, 1972).
[c] Calculated using Crt values for JHM-infected L-2 cells that have been previously published (Cheley *et al.*, 1981b).

The JHM-specific RNA pattern in the brain from 5JWF9 was only visible by RNA transfer analysis after prolonged exposure of the autoradiogram. This sample contained approximately 0.001% of the JHM-specific RNA per unit weight of sample that is present in JHM-infected L-2 cells (Table 3; Cheley *et al.*, 1981b) and contained an amount of JHM-specific RNA that is close to the lower limit of detectibility by our RNA transfer analysis. Hybridization analysis also indicated that the spinal cord tissue from rat 5JWF9 contained more JHM-specific RNA per unit weight of sample than did brain samples from the same animal (Table 3).

Thus we could detect JHM-specific RNA sequences in brains and spinal cords of ic inoculated Wistar Furth rats up to approximately 20 days pi using RNA transfer techniques, and in some cases we could detect viral RNA at least 28 days pi using hybridization kinetics. However, there was generally less JHM-specific RNA in brains and spinal cords in rats sacrificed after 20 days pi than in rats killed before 20 days pi (Tables 2 and 3). In addition, rats inoculated at 10 days of age and killed after 20 days pi had more JHM-specific RNA per unit weight of sample remaining in their spinal cords than in their brains (Table 3).

DISCUSSION

We detected variation in JHM-specific RNA that was present in the brains and spinal cords of Wistar Furth rats which were inoculated ic with JHM at 5-10 days of age. We detected 1-8 JHM-specific RNA species in these Wistar Furth rats (Figs. 1 and 2). All samples with detectable JHM-specific RNA contained the 0.8×10^6-Da JHM nucleocapsid mRNA species; in addition a $2.1\text{-}2.4 \times 10^6$-Da JHM RNA species was detected in all the spinal cord samples tested and all but one of the brain samples (Figs. 1 and 2). The $2.1\text{-}2.4 \times 10^6$-Da RNA species is of interest since it was consistently present in infected Wistar Furth rats as a major viral RNA species but not in JHM-infected L-2 cells. However, this species may be visible as a minor species in *in vitro* JHM infections (Cheley *et al.*, 1981a, b). Two possible sources of this RNA species are (1) the result of a specific processing of a higher-molecular-weight RNA species; (2) the premature termination in the synthesis of the viral E2 glycoprotein mRNA. What role, if any, this viral RNA species plays in the demyelination process remains to be determined.

The variation in JHM-specific RNA seen with individual rats differed markedly with what was observed with *in vitro* JHM infections; thus individual JHM infections of L-2 cells gives a consistent pattern of JHM-specific RNA species (Cheley *et al.*, 1981a, b). One possible explanation for these results would be that infection of different cells in the brain and spinal cord with JHM virus produces different patterns of JHM-specific RNA species. Thus the observed patterns of JHM-specific RNA seen in different brain and spinal cord samples could vary depending on which cells are predominately infected for a given animal. Dubois-Dalcq *et al.* (1982) have reported that JHM can infect both neuronal and nonneuronal cells in C57BL mouse spinal cord cultures and that neuronal cells produce the E2 glycoprotein while infected nonneuronal cells do not. Another explanation for the variation in the viral RNA patterns, which is not mutually exclusively of the first possibility, would be due to selection of viral mutants. Lai *et al.* (1983) has reported that JHM virus isolated from persistently infected neuroblastoma cell lines diverged more markedly than the corresponding virus maintained under the conditions of lytic infection.

Using an RNA transfer technique we detected JHM-specific RNA in the brain and spinal cord samples of rats examined less than 20 days pi. After 20 days pi we could no longer detect virus-specific RNA using this technique. Hybridization kinetic analysis showed that JHM-specific RNA was still detectable in a spinal cord sample taken at 28 days pi (Table 3). In addition, our data indicated that infected brain or spinal cord samples contain only a small fraction (approximately 0.0005-0.003%) of the amount of JHM-specific RNA per unit weight of sample present in *in vitro* infected cells (Table 3). This result suggested

that a relatively small percentage of cells in the spinal cord or brain are infected with the virus. This result is consistent with ultrastructural studies that demonstrated JHM particles only in oligodendrocytes of individual Wistar Furth rats killed at 12-24 days pi (Sorensen *et al.*, 1980). Furthermore, in immunofluorescence studies on JHM-inoculated rats with subacute demyelinating encephalitis, JHM antigen was detected in glial cells of the white matter in the cerebrum and spinal cord but not in neurons (Nagashima *et al.*, 1978). In Wistar Furth rats inoculated ic at 10 days and sacrificed after more than 20 days pi we could detect JHM-specific RNA by hybridization kinetics in the spinal cord but not in the brain. Thus, the viral RNA persisted longer in the spinal cord than in brain samples in these rats; this result suggests that an active viral infection is taking place in the spinal cord after the infection of the brain has ceased or is severely curtailed.

Our results thus raise several interesting questions such as the reason for the heterogenous JHM RNA patterns in inoculated rats and the origin and possible role in demyelination of the JHM-specific $2.1\text{-}2.4 \times 10^6$-Da RNA species. We plan to expand these studies using molecular cloning and other techniques.

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