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Neurovirulence of six different murine coronavirus JHMV variants for rats

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Summary

Six variant viruses of the JHMV strain of murine coronavirus with large (cl-2, CNSV, DL and DS) or small (sp-4 and JHM-X) S proteins were compared in terms of their relative neurovirulence in weanling Lewis rats. Inoculation of various doses of the variants revealed that the cl-2 and CNSV were highly virulent and DL and DS were low-virulent, while sp-4 and JHM-X were avirulent. Pathological examination of rats infected with variants cl-2, DL and sp-4 showed that the cl-2 and DL induced severe and mild acute encephalomyelitis, respectively, while no lesions were observed in the central nervous system of rats infected with sp-4. Virus growth and distribution of antigen in rat brains correlated strongly with neurovirulence. These results suggest that S protein plays a role in neurovirulence in rats. In addition, these variant viruses were shown to be useful tools for further analysis of JHMV neurovirulence in animals as well as in cultured cells.

Murine coronavirus JHM; Neurovirulence; S protein

Introduction

Murine coronavirus JHM strain (JHMV) is known to cause a broad spectrum of central nervous system (CNS) diseases in rodents (Nagashima et al., 1978a; Soren-

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sen et al., 1980, 1984; Knobler et al., 1981; Stohlman and Weiner, 1981; Wege et al., 1981; Koga et al., 1984). In addition to acute encephalomyelitis (AE), intracerebral inoculation of JHMV into the rat CNS produces subacute demyelinating encephalomyelitis (SDE) which has been studied as a model of virus-induced demyelination (Nagashima et al., 1978b; 1979). SDE has been suggested to be triggered by a persistent viral infection (Wege et al., 1984a), and is associated with autoimmune reactions against myelin basic protein (Watanabe et al., 1983, 1987; Massa et al., 1986a).

The JHMV S protein forms the projections protruding from the virion surface. This protein is encoded by mRNA3 and N-linked glycosylated with a molecular weight of 150–180 kDa; it is cleaved cotranslationally into the ca. 90 K S1 and S2 subunits. The S protein has been shown to have some important biological activities, e.g., cell fusion, attachment to receptor on susceptible cells, and elicitation of neutralizing antibodies (Holmes et al., 1981; Collins et al., 1982; Wege et al., 1984b). The S protein is also believed to be a critical determinant in the pathogenesis of the CNS diseases caused by JHMV infection, since JHMV variants selected by monoclonal antibodies against the S protein have marked alterations in neurovirulence and the ability to induce demyelination (Dalziel et al., 1986; Fleming et al., 1986, 1987; Wege et al., 1988).

Recently it was shown that JHMV variants with a large S protein are selectively propagated after inoculation with JHMV with a small S protein in both in vivo rat brains (Taguchi et al., 1985) and in vitro neural cell cultures (Taguchi et al., 1986). These facts suggest that large S proteins play an important role during viral replication in rat brain cells. More recently, it was shown that 4 JHMV variants, cl-2, CNSV, DL, and DS, have large S proteins and 3 distinct antigenic domains recognized by a panel of monoclonal antibodies, while 2 other JHMV variants, sp-4 and JHM-X, have small S proteins and lack 2 of 3 antigenic domains (Taguchi and Fleming, 1989). This clearly showed that 6 different JHMV variants are divided into 2 groups with respect to the size and antigenicity of S proteins. It is of interest to investigate the neurovirulence of JHMV variants with marked antigenic differences in S protein. In this paper, we describe the neurovirulence in rats of 6 different JHMV variants which have been shown to have an antigenically different S protein and discuss the implication of S protein in rat neurovirulence.

Materials and Methods

Virus preparation and titration

The JHMV variants, cl-2 and CNSV, were isolated as described previously (Taguchi et al., 1985, 1986). The DL and DS strain (Stohlman et al., 1982) were kindly provided by Dr. S.A. Stohlman, University of Southern California. JHM-X (Makino et al., 1984) was kindly provided by Dr. S. Makino at the same University. Sp-4 was a recloned virus from wt-JHMV (Taguchi and Fleming, 1989). The stock viruses were propagated and assayed on DBT cells as reported previously (Taguchi et al., 1980).

Animals and virus inoculation

Four-week-old Lewis rats serologically free from MHV infection were purchased from Charles River Japan (Atsugi, Japan). Under anesthesia with ether, the rats were intracerebrally (i.c.) inoculated with varying amounts of each variant, in a final volume of 0.05 ml. Infected animals were observed for 2–4 weeks after inoculation to check the morbidity and mortality. The 50% infectious dose (ID_{50}), namely a dose which induces clinical signs in 50% of inoculated rats and the 50% lethal dose (LD_{50}) were calculated by the method of Reed–Muench.

Titration of infectious virus in the brain and histology

The rats inoculated with 1×10^4 plaque forming units (PFU) of the variants cl-2, DL, and sp-4 were periodically euthanized for autopsy. In each rat, one cerebral hemisphere, removed for titration of infectious virus, was homogenized in 5 ml of Dulbecco's modified Eagle's medium and plaque assayed as described elsewhere (Taguchi et al., 1980). The remaining hemisphere and spinal cord were collected for histopathological examination and were finally stained with hematoxylin–eosin (HE) or HE–luxol fast blue.

Immunohistochemistry

The biotin–streptavidin (B-SA) amplified method was employed for examination of viral antigen on paraffin sections. Briefly, after deparaffinization, sections were treated with trypsin (Hondo et al., 1982; Van Noorden and Polak, 1983) and thereafter with blocking reagent (Boehringer Mannheim, Mannheim, F.R.G.). The sections were then incubated with rabbit anti-JHMV antibody (1:400), that had been kindly provided by Dr. K. Yamaguchi, overnight at 4°C, and endogenous peroxidase was blocked by treatment with 0.3% H_2O_2 in methanol. Subsequently, StrAviGen™ B-SA immunoperoxidase was added (Biogenex Labs., Dublin, CA). After rinsing, peroxidase was developed with 0.02% 3-amino-9-ethylcarbazole (Sigma) and 0.03% H_2O_2 in 0.05 M acetate buffer (pH 5.0) (Van Noorden and Polak, 1983). The sections were counterstained with hematoxylin.

Results

In vivo virulence estimation of 6 JHMV variants

In 2 independent experiments, 4-week-old Lewis rats were inoculated i.c. with 1×10^5 PFU of each variant and observed for 3 weeks after infection. The virus variants fell into 3 clearly distinct virulence groupings as measured by morbidity (i.e., the rate of expression of symptoms in infected rats) and mortality shown in Table 1. It was shown that the cl-2 and CNSV strains were highly virulent, the DL

TABLE 1

Mortality and morbidity of rats inoculated with JHMV variants

	JHMV variants inoculated					
	cl-2	CNSV	DL	DS	sp-4	JHM-X
Exp. 1	3/4/5 ^a (9-12) ^b	3/4/5 (10-12)	1/1/5 (12)	0/0/5	0/0/5	0/0/5
Exp. 2.	6/6/8 (9-14)	5/6/7 (11-14)	1/1/6 (7)	2/2/6 (10-15)	0/0/8	0/0/6
Total						
mortality	69%	67%	18%	18%	0%	0%
Total						
morbidity	77%	83%	18%	18%	0%	0%

Four-week-old Lewis rats were intracerebrally inoculated with 1×10^5 PFU of each variant and observed for 3 weeks after infection.

^a No. of dead/No. of diseased/No. of tested.

^b Time to death in days.

TABLE 2

Mortality and morbidity of rats inoculated with various doses of JHMV variants

Virus	PFU inoculated	Time to death ^a	Mortality and morbidity ^b	
			2	4
cl-2	10^5	14-16 (15)	1/5/5	3/5/5 ^c
	10^4	7-14 (10.3)	3/4/5	3/4/5
	10^3	8-9 (8.3)	4/4/5	4/4/5
	10^2	7-12 (9.5)	2/2/5	2/2/5
CNSV	10^5	8-9 (8.5)	2/5/5	2/5/5
	10^4	7-14 (10.3)	4/5/5	4/5/5
	10^3	8-9 (8.3)	3/5/5	3/5/5
	10^2	14-17 (15.5)	1/5/5	2/5/5
DL	10^5	14	1/2/5	1/2/5
	10^4	14-27 (23.3)	1/1/5	4/4/5
	10^3		0/0/5	- ^d
DS	10^5	7-10 (8.5)	2/5/5	2/5/5
	10^4		0/0/5	0/3/5
	10^3		0/0/5	0/3/5
sp-4	10^5		0/0/5	-
	10^4		0/0/5	-
JHM-X	10^5		0/0/5	0/0/5
	10^4		0/0/5	0/0/5

Four-week-old Lewis rats were intracerebrally inoculated with various titers of variants.

^a In days with mean value in parentheses.

^b No. of dead/No. of diseased/No. of inoculated examined at 2 and 4 weeks p.i.

^c One rat showed a recovery from paralysis.

^d -, not done.

TABLE 3

ID₅₀ and LD₅₀ of JHMV variants for weanling rats

Virus	ID ₅₀ ^a (log ₁₀ PFU)	LD ₅₀ ^a (log ₁₀ PFU)
cl-2	≧ 2.5	3.3 ^b
CNSV	≧ 1.5	3.4 ^b
DL	≧ 5.0	≧ 5.3
DS	4.5	≧ 5.2
sp-4	≧ 5.5	≧ 5.5
JHM-X	≧ 5.5	≧ 5.5

Four-week-old Lewis rats were intracerebrally inoculated with various titers of variants.

^a Calculated by the method of Reed–Muench from the morbidity and mortality obtained at 2 weeks after infection.

^b The doses of both 100% and 0% mortality were postulated.

and DS strains were moderately virulent and the sp-4 and JHM-X strains were avirulent.

The nature of this virulence was then investigated more precisely as a measure of neurovirulence. Various titers of each variant were inoculated i.c. and rats were clinically observed for 2–4 weeks (Table 2). Clinical symptoms characteristic of AE, appearing between 6–14 days p.i., and SDE, appearing as late as 18–27 days p.i., were noted.

Most of the rats inoculated with either cl-2 or CNSV, regardless of virus dose, showed a pattern of AE with ataxic gait and slight hind-limb paralysis appearing about 1 week p.i. By 2 weeks p.i., they had developed severe hind-limb paralysis and paresis resulting in death. In contrast, more than half of the rats inoculated with either DL or DS showed CNS symptoms characteristic of SDE with ataxic gait that slowly developed into paralysis followed by death within 4 weeks. The sp-4 and JHM-X variants induced no clinical symptoms until 4 weeks p.i.

Neurovirulence of the 6 variants could be classified into 3 groups according to ID₅₀ and LD₅₀. ID₅₀ and LD₅₀ were calculated by the method of Reed–Muench from the results tabulated in Table 2 (Table 3). The cl-2 and CNSV strains were revealed to be highly neurovirulent, the DL and DS strains to be of low neurovirulence, and the sp-4 and JHM-X strains to be aneurovirulent.

Growth in the brain of JHMV variants from different virulence groupings

Four-week-old Lewis rats were inoculated i.c. with 1×10^4 PFU of variants, cl-2, DL, or sp-4, and virus titers in the brain were examined by plaque assay. As shown in Fig. 1, cl-2 was consistently recovered from the brain on 3, 6, and 10 days p.i. with a peak on day 6 p.i. Infectious virus was not consistently recovered from rats inoculated with DL and the titers of DL were generally lower than those of cl-2. In contrast, infectious sp-4 was detected in only one of the animals on day 6 p.i. These results showed that the virus titer in the brain was proportional to neurovirulence, i.e., variants showing high mortality and morbidity grew well in the brain.

CNS histopathology after infection with JHMV variants from different virulence groupings

The central nervous systems of weanling rats inoculated with 3 variants with different neurovirulence, cl-2, DL and sp-4, were histopathologically examined (Table 4). The histopathological changes caused by cl-2 and DL were basically similar to those induced by JHMV infection reported previously (Nagashima et al., 1978b, Watanabe et al., 1987). Several prominent histopathologic changes were observed in cl-2 infection. On day 3, small numbers of mononuclear cells infiltrated to the meninges, but parenchyma was not affected. Inflammatory lesions were frequently found in the whole CNS on day 6. On day 10, circumscribed necrotic lesions and spongy degeneration were observed in the mesencephalon, rhombencephalon and gray matter of spinal cord (Fig. 2). In the white matter of the spinal cord, early demyelinating lesions were noted. On day 13, necrotic lesions were also found in the hippocampus. In the brainstem, myelin destruction was observed in the area close to necrotic lesions. The histopathologic changes caused by DL were less severe than those observed in rats infected with cl-2. No lesions were found in the CNS of rats inoculated with DL on days 3 and 6. On day 10 mild neuronal loss and perivascular infiltration of mostly monocytes were observed in the hippocampus of 1 out of 3 rats. On day 13 focal necrosis of neurons was detected in the hippocampus. In the pons moderate perivascular infiltration of mononuclear cells, glial nodules, and glial proliferation were found. No virus-specific lesions were observed in the CNS of rats inoculated with sp-4 throughout the experimental period.

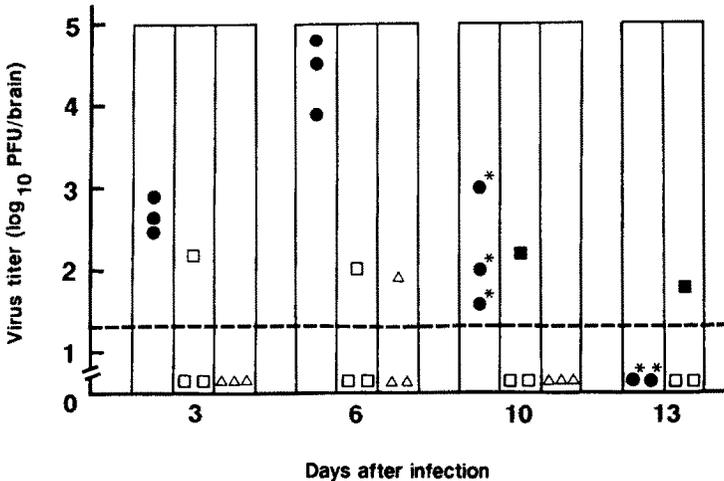


Fig. 1. Growth of JHMV variants, cl-2 (○), DL (□), and sp-4 (△), in rat brains was examined after injecting 1×10^4 PFU of each virus intracerebrally into 4-week-old Lewis rats. Virus titers were determined as described previously (Taguchi et al., 1980). Dotted line indicates the lowest level for detection. Closed symbols represent rats in which viral antigen was detected immunohistochemically. Asterisks represent rats with clinical signs.

TABLE 4

Distribution of lesions in the CNS of rats inoculated with JHMV variants

CNS tissue	Distribution of lesions in rats inoculated with							
	cl-2				DL			
	3	6	10	13	3	6	10	13
Cerebral cortex	-	+	+ ~ ++	+	-	-	-	-
		(2/3)	(3/3)	(1/2)				
Hippocampus	+	++	+	+++	-	-	+++	++
	(3/3)	(3/3)	(3/3)	(2/2)			(1/3)	(1/3)
Di- and mes- encephala	-	+	+	++	-	-	-	-
		(2/3)	(1/3)	(1/2)				
Cerebellum	-	+ ~ ++	+ ~ +++	+++	-	-	-	+
		(3/3)	(3/3)	(2/2)				(1/3)
Pons and myelencephalon	-	++	+++	+++	-	-	+	+++
		(3/3)	(3/3)	(2/2)			(1/3)	(1/3)
Spinal cord gray matter	-	+	++ ~ +++	++	-	-	-	+
		(3/3)	(3/3)	(2/2)				(1/3)
white matter	-	±	++	++	-	-	-	-
		(3/3)	(3/3)	(2/2)				
Meninges	+	++	± ~ +	+	-	-	+	+
	(3/3)	(3/3)	(3/3)	(2/2)			(1/3)	(1/3)

Four-week-old Lewis rats were intracerebrally inoculated with 1×10^4 PFU of each variant.

-, none; ±, slight; +, mild; ++, moderate; + + +, severe.

(No. of affected/No. of examined).

Immunohistochemistry

The distribution of viral antigen was examined by the B-SA amplified method on paraffin sections using polyclonal anti-JHMV antibody. The results of these experiments are summarized in Table 5. Viral antigens were detected in all cl-2 and some DL inoculated rats, but not in sp-4 inoculated ones. Viral antigens were first observed both in neuronal and glial cells in the telencephalon, especially in the hippocampus, and disseminated to these cells in the rhombencephalon and spinal cord (Fig. 3). On day 13 viral antigens were mostly restricted in the glial cells of the pons and spinal cord.

Discussion

We have compared the neurovirulence for rats of 6 different variants of JHMV which have been shown to be divided into 2 groups with respect to the size and antigenicity of S proteins (Taguchi and Fleming, 1989). The cl-2 and CNSV variants with large S proteins isolated from the rat brain and neural cell cultures, respectively

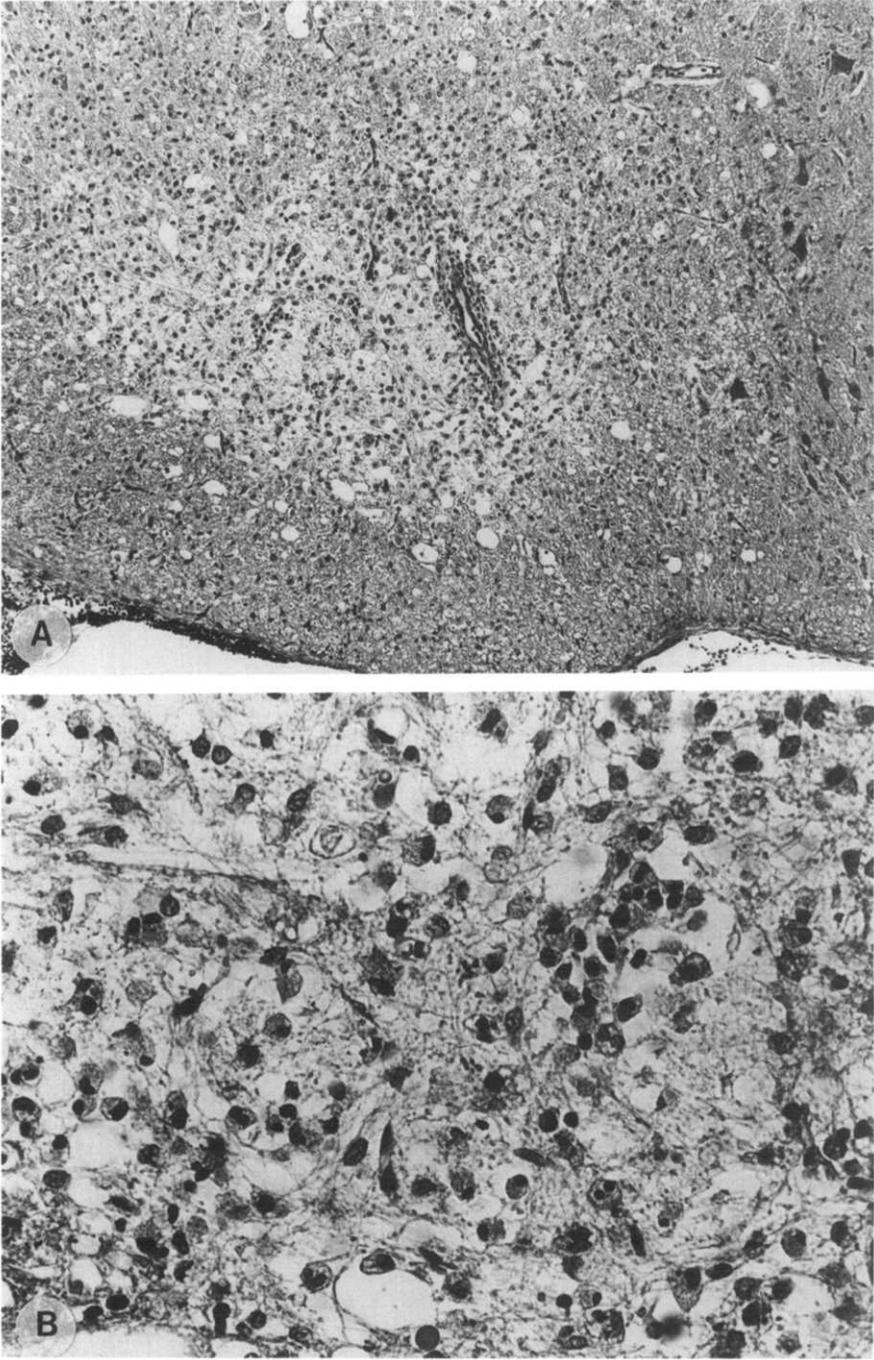


Fig. 2. The pons of rat observed on day 10 after infection with the cl-2. Circumscribed necrotic lesion and perivascular infiltration of many mononuclear cells were found. HE + luxol fast blue, $\times 100$ (A). High magnification of A, many foamy macrophages and phagocytic cells infiltrated (B), $\times 200$.

TABLE 5

Distribution of viral antigen in the CNS of rats inoculated with JHMV variants

CNS tissue	Distribution of viral antigen in rats inoculated with							
	cl-2				DL			
	3	6	10	13	3	6	10	13
Cerebral cortex	N, G ^a (1/3)	N, G (2/3)	G ^a (1/3)	-	-	-	-	-
Hippocampus	N, G (3/3)	N, G (3/3)	-	-	-	-	N ^a , G ^b (1/3)	-
Di- and mes- encephala	N, G ^a (1/3)	N, G (3/3)	N, G ^b (1/3)	G ^a (1/2)	-	-	-	G ^a (1/3)
Cerebellum	-	N, G (2/3)	N, G ^b (3/3)	-	-	-	-	N ^a , G ^a (1/3)
Pons and myelencephalon	-	N, G (3/3)	N, G ^b (3/3)	G ^a (2/2)	-	-	-	G (1/3)
Spinal cord gray matter	-	N, G (3/3)	N, G ^b (3/3)	G ^a (2/2)	-	-	-	G ^a (1/3)
white matter	-	G ^a (2/3)	G (3/3)	G (2/2)	-	-	-	-
Meninges	-	-	-	-	-	-	-	-
Ependyma	-	-	-	-	-	-	-	-
Choroid plexus	-	-	-	-	-	-	-	-

Four-week-old Lewis rats were intracerebrally inoculated with 1×10^4 PFU of each variant. Viral antigen was detected by B-SA amplified method with rabbit anti-JHMV antibody.

Cell types: N, neuron; G, glia.

-, not found. (No. of detected/No. of examined).

^a Noted only rarely.

^b Viral antigen were more abundant in glia than in neurons.

(Taguchi et al., 1985, 1986), were highly virulent. Variants DL and DS also with large S proteins similarly caused severe neurological diseases, although the degree of neurovirulence of these variants was somewhat less than that of the cl-2 and CNSV. In contrast, the sp-4 and JHM-X with small S proteins failed to cause any clinically apparent CNS diseases as long as 4 weeks after inoculation. The cl-2 and DL replicated both in neuronal and glial cells, causing histologically severe AE and mild AE characterized by circumscribed necrotic lesions and necrosis of neurons, respectively, while neither viral antigens nor the lesion caused by the virus infection was detected in the CNS of rats inoculated with sp-4. These observations support the idea that a large S protein is indicative of high viral growth capability in rat brain and that this protein plays an important role in neurovirulence. Recently, it was reported by Morris et al. (1989) that a viral variant with a small S protein, AT11, was isolated from rat spinal cord that had been infected only with large S protein-containing, wild-type JHMV. Interestingly, AT11 cord virus was less neurovirulent for 10-day old Wistar rats than other isolates containing large S protein. This observa-

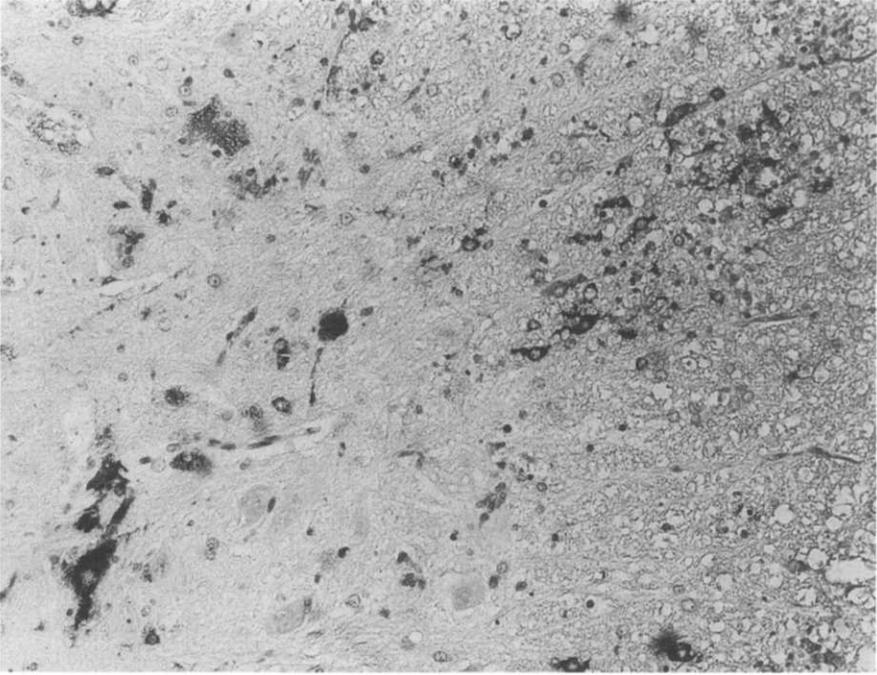


Fig. 3. The ventral area of cervical cord in the rat observed on day 6 after infection with the cl-2. Large amount of viral antigen was detected in neuronal and glial cells. Anti-JHMV immunohistochemistry, $\times 200$.

tion agrees very well with our data which strongly suggest that a large S protein is requisite for effective neurovirulence in rats.

Consistent with this are the results from earlier studies in mice which also suggest that S protein is a critical viral determinant of neurovirulence. For example, variant viruses selected for resistance to neutralization with a monoclonal antibody recognizing a site designated E2(B) have diminished lethality in mice (Fleming et al., 1986). On the other hand, neutralization-resistant variants sequentially selected by monoclonal antibodies recognizing E2(B) and E2(A) have diminished ability to cause demyelination (Fleming et al., 1987). A recent report showed that the determinants recognized by monoclonal antibodies, that is, E2(A) and E2(B), are antigenically altered or deleted in variants with small S proteins (Taguchi and Fleming, 1989). In this study, such variants with small S proteins were shown to be avirulent for weanling rats. These findings suggest that the domains retained on large S protein were key determinants of neurovirulence for rats. Variants DL and DS containing the determinants, E2(A) and E2(B), induced neurological diseases, although their degrees of neurovirulence were not so high as those of the cl-2 and CNSV. It seems likely that the intermediate virulence of DL and DS for rats is related to discrete mutations in large S protein. To locate the S domains specifically present in large S protein, we will obtain cDNA of large mRNA₃ and compare it with cDNA of small mRNA₃ (Schmidt et al., 1987).

Previous studies on the acute phase of JHMV infection in rats, in which the distribution of viral antigen was determined by immunofluorescence, showed evidence of JHMV replication both in glia and neurons, particularly those of the hippocampal region (Nagashima et al., 1978a; Koga et al., 1984; Sorensen et al., 1984; Sorensen and Dales, 1985). The present study confirms these earlier findings and shows that the hippocampal neurons and glia are likely to be early targets. It has been reported that hippocampal neurons, Purkinje neurons of the cerebellum (Sorensen and Dales, 1985) and oligodendrocytes (Sorensen et al., 1980; Wege et al., 1981) are the primary target of JHMV infection and involvement of neurons as a primary target is important for the development of JHMV-induced encephalomyelitis (Sorensen et al., 1982, 1987).

In contrast to this, previous studies of *in vitro* rat brain culture showed that the main target cell for JHMV may be oligodendrocytes (Beushausen and Dales, 1985; Beushausen et al., 1987), type I astrocytes, or brain macrophages (microglia) (Massa et al., 1986b) and neurons have been reported to be resistant to direct infection of JHMV (Massa et al., 1986b). These facts might indicate that neurons are actually primary target cells in the brain, however, such feature of neurons failed to be reproduced in culture. Alternatively, neurons could be infected by JHMV as a result of fusion with infected astrocytes, oligodendrocytes, or microglia in the rat brain. The CNSV and cl-2 variants, which were highly virulent for rats as shown in this study, can multiply in primary rat glial cell cultures consisting of more than 95% astrocytes, while avirulent sp-4 fails to multiply (Taguchi et al., 1986). These findings suggest that JHMV infection in astrocyte is an important factor in inducing the CNS disease. This concept is also supported by a recent study which indicated that the growth properties of JHMV variants are markedly different in the infection of primary rat glial cell cultures and these differences correlate with the disease pattern in animals (Massa et al., 1988).

It was previously reported that only viruses with large S proteins are selectively propagated in weanling rats inoculated with wild-type (wt) JHMV which has a small S protein (Taguchi et al., 1985). It was not clearly understood whether virus mutation occurred in every case in the rat brain after intracerebral inoculation or it was present as a minor undetectable contaminant in the original inoculum which increased due to selection in rat brain. Subsequently, it was found that differences in plaque morphology distinguished wt-JHMV with small S protein from 'cl-2 type' virus with large S protein. If the cultivation time for plaque assay using DBT cells was prolonged from 2-3 or 4 days, the plaques produced by cl-2 had clearer margins and slightly greater diameters than those produced by wt-JHMV. It was occasionally observed that plaques produced by wt-JHMV consisted of mainly 'wt' morphology but a few of them, less than 0.5%, were 'cl-2 type' plaques. One plaque with a clear 'cl-2 type' morphology was studied in detail and was found to have viruses which contained large mRNA₃ (data not shown). This finding implies that the wt-JHMV used in previous studies contained a very minor subpopulation of 'cl-2 type' virus, and it seems likely that this contaminating virus was then selectively amplified in the rat brain. This interpretation is supported by experiments in which after inoculation of plaque-purified wt-JHMV (sp-4) into the rat brain, no rat

showed the CNS disease, and no virus with a large mRNA₃ was recovered (data not shown). Evidence suggests that RNA viruses have a very high mutation rate (Holland et al., 1982), such that it may be difficult to produce virus populations that do not have small numbers of such variants.

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