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In vivo and *in vitro* models of demyelinating disease. XVII. The infectious process in athymic rats inoculated with JHM virus*

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Wistar Lewis (WL), Long Evans (LE) and other rat strains develop complete resistance to CNS disease when inoculated intracerebrally with murine hepatitis JHM virus (JHMV) after the 10th day of age.[1] Two types of studies were conducted to ascertain the involvement of the cellular immune system in development of resistance. Immunosuppression of WL rats with cyclosporin A (CsA) following onset of the age-related resistance demonstrated that this drug was partially able to abrogate resistance. In the other studies nude (rnu/rnu) rats, their heterozygous (rnu/+) litter mates and genetically related LE rats of various ages were challenged with JHMV. The rnu/+ and LE animals became completely resistant before the age of weaning, whereas some rnu/rnu rats, challenged as late as 70 days of age, showed disease symptoms—albeit after a long latent period. These observations indicated that the cellular immune system plays an important role in suppressing the disease process in the CNS. When the infection of nude rats was initiated on or after the 15th day of life, the histological lesions were generally small and present in both grey and white matter but were seldom seen in the spinal cord. Mononuclear infiltrates were evident throughout the CNS. In some nude rats there was massive mononuclear cell infiltration towards the meningies and into ventricular spaces. By contrast in rnu/+, LE and WL rats with late-onset disease symptoms, demyelinating-type lesions were confined to the white matter and only minor infiltration of mononuclear cells was evident. JHMV RNA was detectable by dot-blotting analysis in the CNS of both paralysed and asymptomatic rnu/rnu and rnu/+ rats, but less RNA was usually detected in heterozygous animals. *In-situ* hybridization with cDNA probes for JHMV RNA showed that neurons in the hippocampus and cerebellum, as well as cells in the white matter, were frequently infected. The present data indicate that in the rat T cells have an important function in maintaining resistance to the JHMV related disease process. However, even without a functional T cell compartment nude rats challenged after 15 days of age did not develop an acute encephalitis, suggesting that an age-dependent, non-immunological mechanism is also involved in restricting the spread of infection. It is possible that resistance in euthymic rats sets in because: (1) at the time of weaning the CNS matures, so that the number of targets available for infection is reduced, (2) T cells prevent the late-onset disease by clearing persistent, low grade infections from the CNS.

Key words: nude rats; coronavirus; CNS disease; demyelination.

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Introduction

Infection of the central nervous system (CNS) of rats with JHM virus (JHMV) may result in a demyelinating disease with a prolonged latency period.[1–3] The rat becomes naturally resistant to intracerebral (i.c.) inoculation with JHMV sometime between birth and weaning. Resistance develops sooner in the Wistar Lewis (WL) and several of the outbred strains tested than in the Wistar Furth (WF) strain.[4] The process of myelination is completed later in the WF strain than in other rat strains, presumably in connection with a slower rate of growth,[5] suggesting existence of a reciprocal relationship between myelination and infectability by JHMV. Consistent with this are observations on primary explants of rat and mouse oligodenrocytes, which indicate that JHMV can replicate in immature, not in differentiated oligodendrocytes.[6,7] Whether the age-related resistance in rats is due entirely to the maturation and differentiation of target cells or whether other factors are involved, such as the host's immune system, required clarification. We had previously shown that immunosuppression of asymptomatic infected rats with cyclophosphamide can exacerbate an inapparent JHMV infection, thereby extending susceptibility beyond the period of weaning.[4] However, intrathecal production of anti-JHMV immunoglobulin in acutely and chronically diseased rats fails to prevent the disease process, suggesting that cellular, rather than humoral immunity, may be a determinant in resistance.[8]

To further define the role of cellular immunity in resistance to JHMV, studies were undertaken on the effect of immunosuppression, with cylosporin A and due to absence of a functional thymus. The observations presented here revealed that both postnatal development and cellular immune responses influence the infection of the rat CNS by JHMV.

Results

The inbred Wistar Lewis (WL) strain and most other rat strains tested become refractory to JHMV challenge within 15 days of age.[7] To determine whether T cell functions are essential to the development and maintenance of resistance WL rats were given 10 daily intraperitoneal injections of CSA. Immunosuppression was commenced on the 15th day after birth and the rats were challenged with virus on the 16th day. Four out of 13 animals in the group treated with CsA became paralysed by the 28th day post-infection and died. Therefore, CsA immunosuppression was effective in abrogating the age related resistance in some animals.

CsA may affect cell-mediated immunity by inhibiting functions of T-helper cells and stimulating those of T-suppressor cells.[9,10] The possibility remained, however that CsA affects some as yet unidentified antiviral responses. Genetically athymic nude (rnu/rnu) rats, provide a system free of these uncertainities. The strain carrying the athymic, nude mutation was selected from animals on a hooded background, genetically related to the Long Evans strain. These genetic interrelationships allowed us to make comparisons between the athymic (rnu/rnu), thymus-containing heterozygous (rnu/+) and euthymic (+/+) LE rats. Both LE and heterozygous rats became resistant to JHMV related disease by the 15th day (Table 1). The nude rats, by contrast, remained susceptible to infection throughout the test period, until the 70th day after birth. However, as the age at inoculation was increased the latent period elapsing until clinical symptoms became apparent was lengthened. Thus rats infected when 21–28 days old developed symptoms 28–35 days later, those challenged at 42–49 days of age manifested paralysis 36–42 days later and the group injected at 63–70 days included one asymptomatic animal which died on the 77th day and another on the

Table 1 Incidence of paralysis and death following intracerebral challenge with JHMV

Rat strain (genotype)	Age at inoculation (days)	Number inoculated	Number of deaths	Number paralysed/number dead[a] days post-inoculation						
				7	14	21	28–35	36–42	43–84	151
Long Evans	2	16	10	0/1	1/5	2/3	0	0	0	1/1
	15	10	1	0	0	0	0	1/1		
	21	10	0							
	30	7	0							
Heterozygous	5	7	4	0	0	4/4				
Nude	15	6	0							
(rnu/+)	21–28	6	0							
	42–49	5	0							
Homozygous	5	1	1	0	0	0/1				
Nude	15	3	3	0	0	0	3/3			
(rnu/rnu)	21–28	15	13	0	0	0	4/5	2/2	6/6	
	42–49	6	5	0	0	0	0	3/3	1/2	
	63–70	4	2	0	0	0	0	0	0/1	0/1

[a] Rats which died or killed *in extremis*.

151st day after virus challenge (Table 1). None of the nude rats inoculated at or beyond 15 days of age developed the rapidly fatal, acute encephalomyelitis noted when 2–5 day old WF rats are injected with JHMV.[1,4] These results demonstrated that although rnu/rnu rats were unable to completely resist JHMV they could, like their rnu/+ and +/+ genetic relatives, become resistant within one week after birth to an acute form of rapidly disseminated encephalomyelitis. The current data, therefore, indicated that T cell functions may be involved in arresting or limiting the spread of JHMV infection in older rats and suggest that non-immune controls, such as the ability of JHMV to infect oligodendrocytes prior to terminal differentiation,[6,7] are still operative in nude rats.

Unlike athymic mice, which even as adults are infectable by either the i.c., or intraperitoneal (i.p.) and intranasal routes (4), rnu/rnu rats did not develop any clinical symptoms or histopathological indications of disease when challenged i.p. with JHMV. Thus T cell functions are not the prime factor preventing spread of the infection into the CNS of the rat.

Previous data[1,4,8] and results with the LE strain, tested here, indicated that a long incubation period and localization of the paralysis in the hind limbs, are characteristic of the demyelinating CNS disease of rats.[1,4,8] By contrast some nude rats with extended latent period (Table 1), developed paresis or paralysis in both front and hind limbs. This observation presumably indicated occurrence of more extensive demyelination in these animals. However, not all rats affected by JHMV inoculated at 42–49 and 63–70 of age were paralysed at death (Table 1).

The histopathology observed on examination of CNS tissues from infected LE and rnu/+ rats closely resembled that described by us previously for WF and WL rats.[1,4,11] The extent of tissue damage, degree of involvement of grey and/or white matter and latency period prior to onset of symptoms were correlated with the age at inoculation, as has been previously reported.[1] In rnu/rnu rats manifesting the late-onset disease symptoms, numerous lesions were present throughout the CNS. One nude animal which succumbed by the 19th day, after inoculation as a 5 day old, manifested extensive grey and white matter necrosis throughout the CNS, as well as infiltration

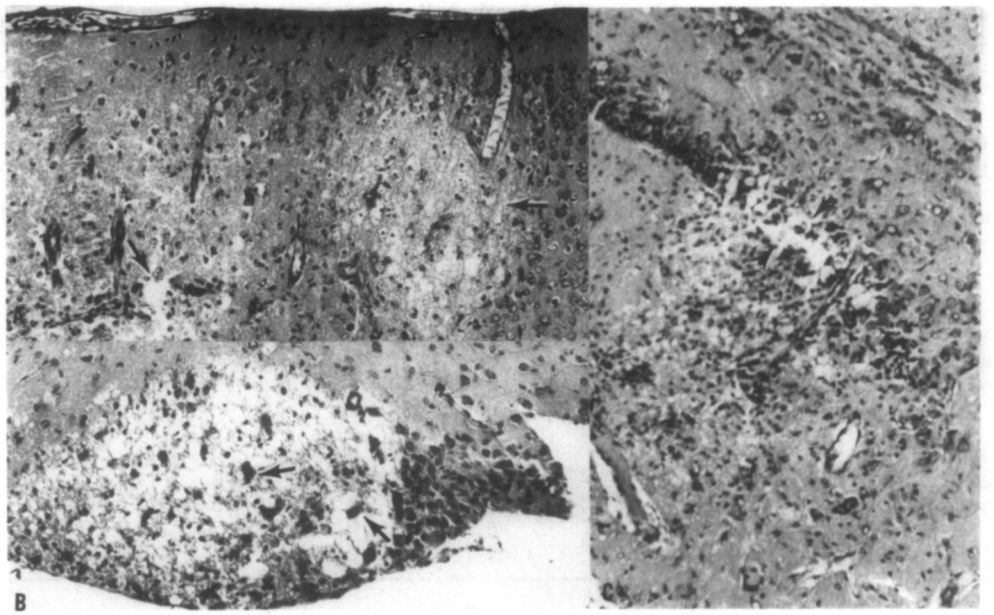

Fig. 1. Selected areas of brain of nude rats injected with JHMV, shown to illustrate histopathology. (a), (b) from a rat inoculated at 5 days of age and killed 19 days later. In (a) an area of the telencephalon with 2 large lesions (arrows), and small, dense infiltrating mononuclear cells. Normal tissue is evident in the upper right hand corner. (b) A section through the optic chiasma showing extensive tissue degeneration and demyelination of axons. Several syncytia, are indicated by arrows. (c) An area of the hippocampus of a rat inoculated at weaning and killed 23 days later. Note the neuronal destruction (arrowheads), vacuolation of the neuropile and presence of numerous infiltrating mononuclear cells. Magnifications: (a) ×225, (b) ×240, (c) ×410.

of mononuclear cells into the neuropile (Figs 1(a) and (b)). Lesions noted in rats inoculated when 15 days of age or older were smaller and more circumscribed (Fig. 1). In rats with latent periods of 7–12 weeks until onset of symptoms, the only histological indications of CNS infection were prominent mononuclear infiltrates into the neuropile (Figs 2(a)–(c)). Demyelinating lesions in the spinal cord, of the type observed in paralysed LE and rnu/+ rats, were not found in rnu/rnu rats. Demyelination was, however, apparent in the cerebellum and myelencephalon, as illustrated in Figs 3 and 4. Large cell syncytia, as seen in Fig. 4(a), have been associated with coronavirus infections *in vivo* and *in vitro*.

The cellular infiltrates noted in the myelencephalon, cerebellum and spinal cord were usually quite small (Figs 2 and 3(a)), although more extensive deposits were occasionally observed, as shown in Fig. 5. However, in the meninges (Fig. 6(a) and (b)) and in the ventricles of the brain (Fig. 6(c)) of some nude rats very massive cell deposits could be observed. The origin of these cells and the stimulus inducing their infiltration into the CNS remain to be elucidated. Such extensive memingeal and ventricular infiltration has not been observed in any other rat strain following challenge with JHMV.

In summary, the histopathology observed in nude rats undergoing chronic JHMV infections differed from that evident in both acutely and chronically infected LE rats, examined here and in other strains, previously studied by us.[1] Particularly notable was the continued destruction of susceptible CNS neurons in the late-onset disease of rnu/rnu animals, not observed in euthymic rats. With the late-onset disease, which is produced after JHMV infection in animals possessing an active thymus, the CNS lesions were restricted to myelinated tracts of the spinal cord and myelencephalon,

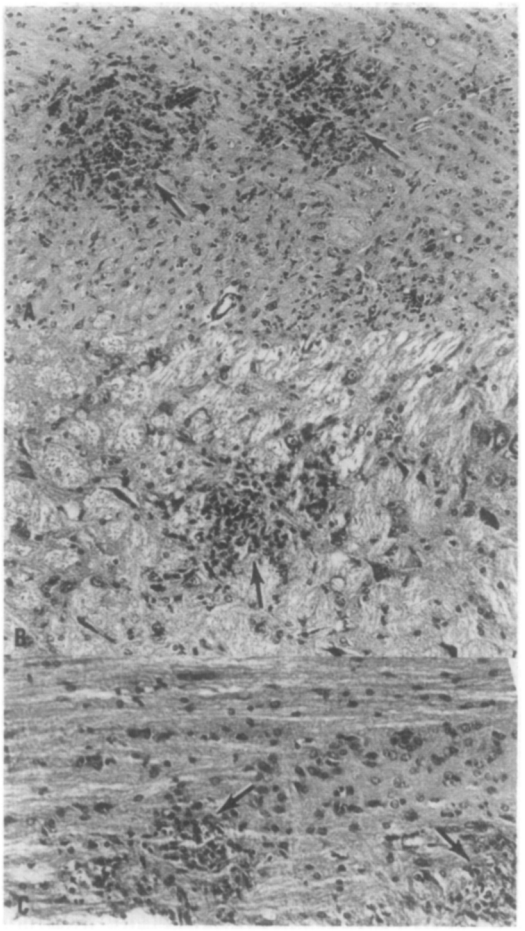

Fig. 2. Brain and spinal cord from a nude rat inoculated at weaning, which showed symptoms of disease about 9 weeks post inoculation. Selected regions of (a) myelencephalon, (b) and (c) spinal cord demonstrate presence of extensive cellular infiltrates (arrows) without apparent destruction of the neural tissue. In (c) the upper area, normal myelination is evident. (a) ×290, (b) ×465, (c) ×300.

whereas in athymic rats, regardless of the interval between inoculation and manifestation of disease, both grey and white matter lesions were formed.

Dissemination of JHMV throughout the CNS of rnu/rnu and rnu/+ rats was ascertained using tissue samples from specified regions of brain and spinal cord, as previously described.[8] When total RNA was extracted, dot-blotted onto nitrocellulose and probed with a JHMV-specific cDNA it was evident that viral RNA was spread throughout the CNS, of paralysed nude rats, even in individual animals inoculated as late as 3–4 weeks of age and killed 46–72 days later (Fig. 7(a)). In the spinal cord RNA was usually either absent or much less in amount. Tissue from three paralysed nude rats, infected when about 6 weeks old and one paralysed rat, infected at 9–10 weeks of age, also contained viral RNA in the telencephalon, mesencephalon, cerebellum, myelencephalon and occassionally in the cervical spinal cord (Figs 7(b) and (c)). Low levels of JHMV RNA were detectable in restricted CNS regions of a nude rat inoculated when 10 weeks old, which had remained asymptomatic for 173 days and in a heterozygous rat challenged with virus at the time of weaning, which had remained without disease symptoms until it was sacrificed 163 days later (Fig. 7(d)). Thus correlative evidence was obtained from dot-blots and histopathological studies, indicating that the infectious process can be initiated in nude rats beyond the

Fig. 3. Extensive destruction of myelinated tissue is apparent in another nude rat inoculated at weaning and killed 9 weeks later. In (a) section through the cerebellum, where part of the 'granular layer' is evident on the upper left. Presence of lymphatic infiltrates is indicated by arrows. (b) A section through the myelencephalon. Perimeter of a large lesion is indicated by arrows. (a) ×240, (b) ×200.

time of weaning, an age at which rats normally have acquired resistance. When the infection of nude animals was initiated late it usually progressed slowly, although it was spread to both grey and white matter. This finding was contrary to the situation evident with the chronic type of disease in euthymic rats, in which lesions are confined predominantly to regions of white matter.[8]

The precise location of infected cells within the CNS was determined by means of *in situ* hybridization using JHMV-specific [^{32}S]-cDNA probes. Whenever possible, analyses of individual rats by dot-blots of RNA extracted from one half of the CNS were correlated with the *in situ* hybridization on sections from the contralateral half. These analyses indicated that hippocampal neurons contained large amounts of viral RNA, as illustrated in Fig. 8(a). Less extensive JHMV RNA labeling was noted in the myelencephalon where silver grains sometimes overlay cells of neuronal morphology, as well as cells which may not be neurons (Fig. 8(b). The RNA was also detected in the white matter of the cerebellum (data not shown). Data which summarizes the findings from dot-blot analyses on nude rats are presented in Table 2. These results clearly show that when challenged with JHMV after weaning these animals underwent a prolonged infection which frequently became widely disseminated through the CNS tissues, regardless whether paralytic disease symptoms were evident or not. Comparisons between findings from dot-blotting and in-situ hybridization with cDNA probes showed that in the 12 rats listed in Table 2 detection of viral RNA extracted from bulk tissue closely paralleled that obtained from tissue sections (data not shown).

Discussion

Evidence from present studies shows that in the rat manifestatons of chronic CNS disease due to JHMV infection, which include virus expression, ataxia, paralysis as

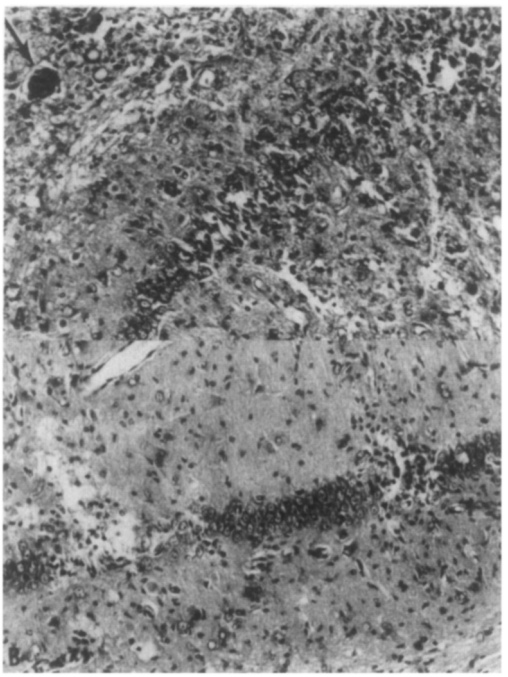

Fig. 4. Presence of lesions in a nude rat, inoculated at 9 weeks of age and killed when about 20 weeks old. In both (a) and (b) extensive destruction of the hippocampal neurons is evident. The arrow in (a) indicates a large syncytium. (a) and (b) ×240.

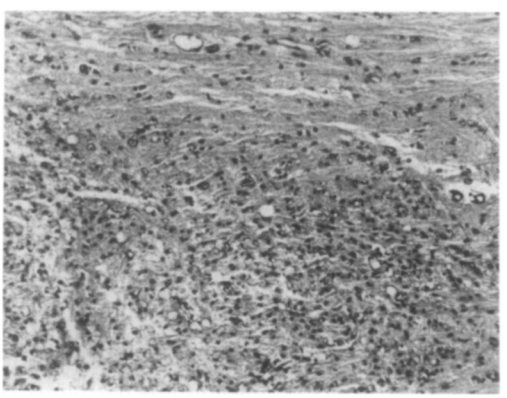

Fig. 5. An area of the myelencephalon from a nude rat inoculated at 9 weeks of age and killed at about 20 weeks of age, with a considerable infiltration of mononuclear cells in the absence of any evidence of demyelinating lesions, ×240.

well as tissue damage in the grey and white matter, may be suppressed by activity of the cellular immune system. Thus age-related resistance to the JHMV-induced demyelinating disease, which develops in euthymic animals before weaning, is abrogated in athymic nude rats. CsA which suppresses T-helper and enhances T-suppressor functions,[9,10] was also able to nullify to a limited extent the age-related resistance of WL rats. However, the immunosuppressant drug cyclophosphamide,

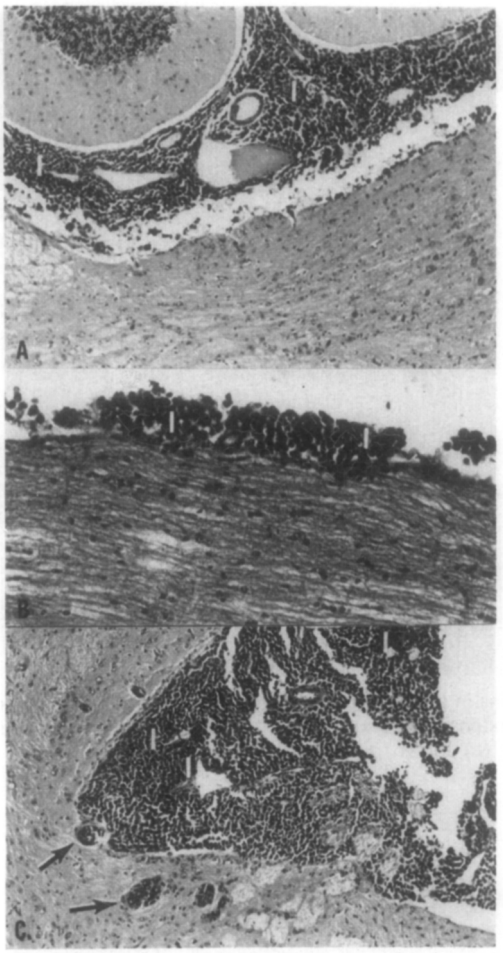

Fig. 6. The meningies in a region of the cerebellum (a) and spinal cord (b) of a nude rat inoculated at weaning and killed 10 weeks later. Extensive infiltration (I) into the meninges by mononuclear cells is evident. In this animal the tissues throughout the CNS appeared to be histologically normal. (c) Lateral ventricle in the vicinity of the dentate gyrus from a nude rat showing extensive infiltration of mononuclear cells into the lateral ventricle and blood vessels (arrows). (a) ×100, (b) ×215, (c), ×170.

which can exacerbate in apparent CNS infections in asymptomatic WF rats,[4] fails to effect a breakthrough in the age-related resistance acquired by WL rats (data not shown), demonstrating that in this model CsA is a more effective immunosuppressant than cyclophosphamide.

To initiate the disease process with JHMV in rnu/rnu rats, inoculation must be given by the i.c. route, whereas intranasal, intraperitoneal as well as i.c. injections can induce CNS disease in athymic mice.[4] In mice, but not in rats, infection can be established in cells outside the CNS compartment, suggesting that in mice surveillance by cellular immunity may prevent spread of the virus into the CNS.

Concerning the histopathological observations associated with the chronic disease process, athymic rats appear to develop lesions in both the grey and white matter, regardless of the length of the incubation period, whereas CNS lesions in euthymic animals with late-onset disease are almost invariably confined to the white matter.[1,4]

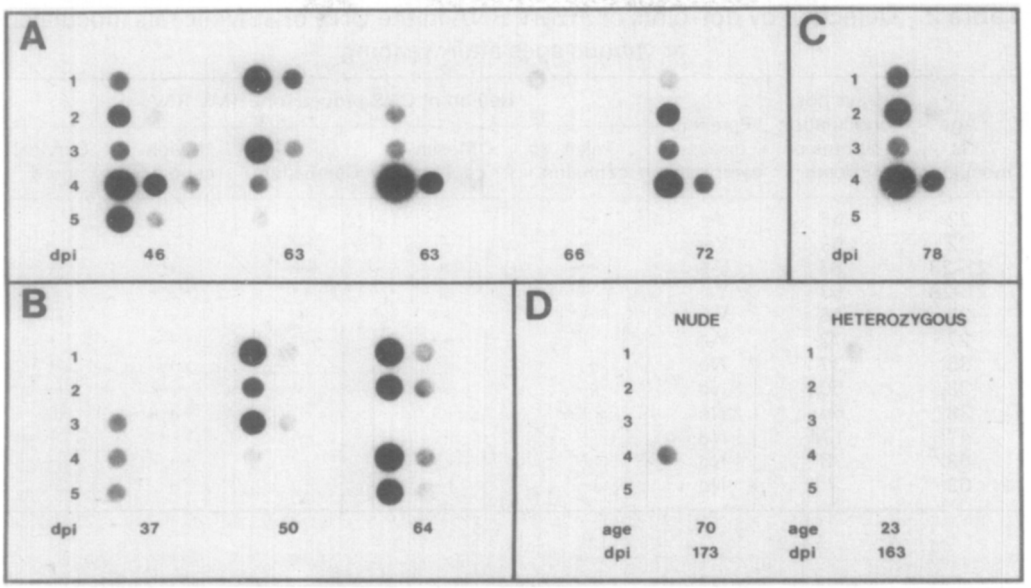

Fig. 7. A composite of dot-blot analyses of the CNS from nude rats inoculated at various ages and killed on different days after inoculation (dpi). The age at inoculation of the (a) group was 21–28 days of (b) was 35–49 days, of (c) was 70 days. In (d) the nude rat was 70 days old when inoculated and remained asymptomatic until it was killed 173 days later. The heterozygous rat was inoculated at 23 days of age and had not manifested symptoms when it was killed 163 days later. Each panel shows the results of probing samples of total RNA extracted from CNS tissues, applied to nitrocellulose in an undiluted form and at dilutions of 1:10 and 1:100. Specific regions of the CNS from which samples were taken are: 1. telencephalon 2. mesencephalon 3. cerebellum 4. myelencephalon 5. cervical spinal cord.

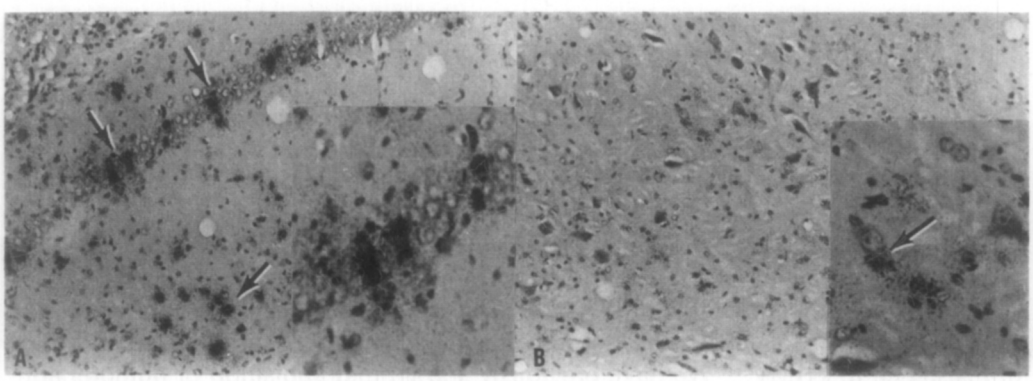

Fig. 8. Selected examples of *in situ* hybridization with [^{35}S]-cDNA probes on tissue sections of chronically infected nude rats inoculated at 38 days of age. (a) A region of the hippocampus of a rat killed 50 days post-inoculation. Silver grains are evident over neurons and cells in adjacent tissue (arrows). Grains over several hippocampal neurons are illustrated at a higher magnification in the inset. (b) The myelencephalon of a rat killed 50 days post-inoculation. Less intense labeling is evident over cells possessing neuronal and non-neuronal (arrow inset) morphology. (a) ×130; inset ×255; (b) ×215, inset ×550.

Nevertheless, hippocampal neurons and the Purkinje neurons of the cerebellum are possible repository sites of latent JHMV in both athymic and euthymic rats[11] (and present data), and thus may be the foci from which widespread infection can be triggered.[4] These observations again emphasize the importance of neurons in development of JHMV-induced encephalomyelitis.

Table 2 Detection by dot-blots of JHMV RNA in the CNS of athymic rats inoculated at various ages after weaning

Age at inoculation	Days post-inoculation at time of death	Paralytic disease symptoms	Region of CNS probed for JHMV RNA				
			Telen-cephalon	Mesen-cephalon	Cerebellum	Mylen-cephalon	Cervical cord
22	66	Yes	+−	+−	+−	−	−
22	69	Yes	−	−	−	−	−
21–28	63	Yes	++	+	++	+	+
21–28	63	Yes	−	+	+	+++	−
27	46	Yes	+	++	+	+++	++
27	72	Yes	+	++	+	+++	+
38	37	No	+−	+−	+	+	+
38	50	No	++	++	++	+−	+−
38	64	No	+++	++	−	+++	++
47	174	No	−	−	−	−	−
63	78	No	+	++	+	+++	−
63	?	No	−	+−	−	+	−

A notable feature of the prolonged disease in nude rats was the infiltration of mononuclear cells into the CNS. Lymphocytic infiltrates were noted as single cells or as small aggregates, in various regions of the CNS, regardless of the presence or absence of neighbouring lesions. The cell infiltrates are reminiscent of typical inflammatory responses associated with experimental allergic encephalomyelitis in the rat and other mammals (see 12 for pertinent references). Infiltration of the meningies and ventricles was also noted occassionally as massive depositions of mononuclear cells. The identity of these cells and the stimulus by which they are mobilized into the CNS have not been established. It is conceivable that they indicate an abortive response by a deficient immune system against the virus infection.

It is also unclear what stimulus might trigger infiltration of the CNS. Vos *et al.*[12] have suggested that in nude rats the T-dependent immune responses are more depressed than those of nude mice, so that mitogens such as PHA, Con A or PWM[12,13] fail to stimulate DNA synthesis in isolated nude rat lymphocytes and exogenous IL-2 does not restore the proliferative activity.[13] Furthermore rnu/rnu lymphocytes do not produce significant amounts of IL-2[13] and the animals are unresponsive to T cell-dependent antigens such as ovalbumin or tetnus toxoid.[12] However, as with nude mice,[14] NK cell activity is increased in nude rats.[15]

The present findings on the influence of cellular immunity upon the chronic disease process need to be related to our previous evidence regarding the age-related aquisition of resistance to JHMV.[1,4] We have previously shown that aquisition of humoral anti-JHMV immunity, localized within the CNS, does not appear to block the spread of the infection.[4] But the fact that inoculation of the virus into athymic rats, even as late as 70 days of age, elicits CNS infections demonstrates that the disease is controlled primarily by the cellular immune system. However, age and strain-related resistance to JHMV are also controlled by the differentiation of oligodendrocyte target cells during the maturation of the rat CNS.[6] Thus cellular immunity may affect clearance of the virus in older rats when differentiation of target cells has already restricted replication to a small subset of immature progenitors. It is clear from previous *in vivo* studies that when rats are challenged late in their susceptible period there is a prolongation of the time elapsing before disease becomes apparent.[1,4] Similarly in athymic rats the development of disease is delayed in older animals, as compared to neonates, perhaps because fewer infectable target cells are present following maturation of the CNS.

Therefore, both maturation of the CNS and cellular immunity probably exert an influence over virus dissemination.

Materials and methods

Virus. JHMV was propagated in L-2 murine fibroblasts and prepared for injection as previously described.[7] At the ages indicated in Table 1 each rat received about 10^5pfu JHMV by intracerebral (IC) inoculation. Uninfected controls were inoculated similarly with fragments of uninfected L-2 cells prepared in the same manner as the virus inoculum.

Animals. Inbred WL rats were purchased from Canadian Breeding Laboratories, St Contant, Quebec and outbred Long Evans rats from Charles River, Montreal, Quebec. Weanling and young adult (150–200 g) homozygous (rnu/rnu) athymic Nude rats were obtained from Harlan Sprague Dawley. Heterozygous (rnu/+) pregnant females, purchased from the same supplier, produced mixed litters of homozygous nude and heterozygous pups. These animals were inoculated i.c. at various ages, as indicated in Table 1.

Histology and immunofluorescence. Animals with clinical indications of neurologic disease were killed and half their brain, segments of spinal cord and portions of spleen, liver, and kidney were fixed in 3:1 ethanol:acetic acid. Following processing, these tissues were employed for histopathology, immunofluorescence examination and *in situ* hybridization with JHMV-specific cDNA probes, as described previously.[8,11]

Immunosuppression. Immunosuppression with cyclosporin A (CsA) (Sandimmune, Sandoz Canada Inc., kindly provided by Dr L. Chow) was accomplished using daily i.p. injections of 25 mg/kg body weight, commencing at 15 days of age. Challenge i.c. with JHMV in this group of rats was at 16 days of age.

Detection of JHMV RNA. JHMV-specific RNA was isolated from infected L-2 cells and used as the template for synthesis of [^{32}P]-cDNA or [^{35}S]-cDNA, as previously described.[8,11] For dot-blot analyses, total RNA in CNS tissue was extracted from different regions of the CNS including the brain and segments of the cervical and lumbar spinal cord, as previously described.[8] Dot-blot analysis was carried out under stringent conditions with [^{32}P]-cDNA, according to.[16]

In situ hybridization with [^{35}S]-cDNA probes was carried out on sections adjacent to those studied by immunofluorescence or histological staining. Tissue sections were collected on glass slides and the hybridization carried out as previously described in.[16]

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