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In vivo and *in vitro* models of demyelinating diseases XXIV. The infectious process in cyclosporin A treated Wistar Lewis rats inoculated with JHM virus*

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In the present study we investigated age related effects of inoculum size and cellular immunity on the CNS disease caused by JHM virus (JHMV) in Wistar Lewis (WL) rats. Onset of resistance normally becomes evident by the 10th day when inoculation is made with 10^5 pfu or less. The resistance could be abrogated in 15 day old animals by increasing the dose two-fold, but with rare exceptions, in 35 day old rats an 80-fold increase in pfu fails to surmount resistance. However, treatment with the immunosuppressant drug cyclosporin A (CsA) abolished resistance, whereby rats challenged at 35 days of age were susceptible to JHMV. The histopathological evidence and disease symptoms in the CsA treated group resembled closely those observed in our previous study with athymic, nude rats. Microscopic examination of the CNS from untreated, infected rats revealed extensive inflammatory responses characterized by perivascular cuffing and mononuclear infiltrates into the neuropile. The parallel CsA treated group showed that inflammatory responses of this type in the CNS were either minimal or absent. From the present evidence, we conclude that JHMV infection, which involves both neuronal and oligodendrocytic elements, is kept in check by the cellular immune system. When cellular immunity is suppressed or absent the disease process is altered from one in which white matter demyelination predominates to another form of disease in which neuronal involvement is prominent.

Key words: cyclosporin A effects; JHM virus infection; CNS disease; rat.

Introduction

Inoculation of inbred rats with defined quantities of coronavirus JHM (JHMV) elicits different types of neurological disease, depending on the age when the virus is inoculated intracerebrally (i.c.). Infection initiated during the first few days of life results in a rapidly fatal, acute encephalomyelitis during which extensive destruction of the grey matter occurs. When older pre-weanling rats are challenged, a delayed, chronic disease frequently develops, which is characterized by progressive paralysis and presence of extensive lesions in the white matter.[1–3] In Wistar Lewis (WL) as well

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as several outbred rat strains tested by us, animals become resistant to JHMV by about the 10th day of life,[3,4] whereas Wistar Furth (WF) rats remain susceptible to the virus until weaning, about 21 days after birth.[3,5] We hypothesized that these age-related differences between the WL and WF strains may be connected with dissimilar growth rates and maturation of the CNS.[6–8] This idea is supported by *in vitro* studies which demonstrated that a relationship exists between oligodendrocyte differentiation/maturation and resistance to infection by JHMV.[9,10]

Another factor controlling neurological disease induced in rats by JHMV is cellular immunity. Rats either immunosuppressed with cyclosporin A (CsA) or genetically athymic remain susceptible to the infection beyond the normal age of resistance, even well past weaning.[4]

To further clarify the role of JHMV and the cellular immune system, in this study we examined the influence of inoculum size, age and CsA immunosuppression on the disease process.

Results

Relationship between inoculum size and age-related susceptibility to JHMV

In several of our previous articles, we reported that WL rats fail to develop disease when inoculated i.c. with 5×10^4 pfu of JHMV by the 10th day after birth.[2,3,11] This finding was confirmed in the present study.

Table 1 Influence of age, size of the inoculum and immunosuppression by cyclosporin A on neurological disease in Wistar Lewis rats

Group	Age at inoculation (in days)	Number of rats affected/total inoculated	Age (in days) of animals with neurological symptoms at time of sacrifice						
			14–21	22–28	29–35	36–43	44–57	58–65	66–73
1a	10	0/2							
1b	10	2/2		1		1			
2a	16	1/8		1					
2b	16	13/16	1	3	5		4		
2c	16	0/4							
3a	15	5/7	1	4					
3b	15	5/5	2	1		2			
4a	35	1/11					1		
4b	35	10/11					5	3	2
4c	35	0/4							
4d	35	0/2							

Groups 1 and 2 were inoculated with 10^5 pfu JHMV; group 3 with 2×10^5 pfu; group 4 with 8×10^5 pfu.

In groups 1a, 2a, 3a and 4a rats were not treated with CsA.

Group 1b received daily CsA injections commencing on the 9th day until the 13th day, when treatment was suspended, then resumed on the 24th day postpartum.

Group 2b received daily CsA injections commencing on the 15th day until they were suppressed on the 25th day and resumed on the 36th day postpartum.

Group 2c were injected daily with CsA commencing on the 26th day, which was 10 days post JHMV inoculation.

Group 3b received daily CsA injections commencing on 14th day of age.

Group 4b received daily CsA injections commencing on 33rd day until they were suspended on the 37th day and resumed on the 48th day postpartum.

Group 4c received CsA daily, commencing on the 49th day which was 14 days post JHMV inoculation.

Group 4d were injected daily with CsA, starting on the 33rd day until 37th day postpartum. In this group CsA treatment was not resumed.

To examine whether resistance prior to and after weaning can be overcome by increasing the virus dose, we challenged one group of 16 day old animals (group 2a in Table 1) with 10^5 pfu, another at 15 days of age (group 3a) with 2×10^5 pfu and a third 35 day old group (4a) with 8×10^5 pfu. It is evident from the data in Table 1 that only one of the eight rats in group 2a developed neurological symptoms whereas five of the seven rats in group 3a, receiving five times more virus at about the same age as 2a, became diseased. Among 4a animals challenged with 8×10^5 pfu JHMV when 35 days old, only one out of 11 developed a late onset, progressive, paralytic disease of the type described by us previously.[2] It is apparent from these data that at 15 days of age inoculation i.c. of about five to 10 times more virus than the standard dose of 5×10^4 pfu may be sufficient to overcome resistance. However, by 35 days, 80 times greater JHMV inocula very occasionally surmounted resistance of WL rats.

Effect of cyclosporin A on age-related resistance to neurological disease caused by JHMV

Our investigation of athymic (rnu/rnu) rats showed that i.c. inoculation of JHMV, even when delayed until the 63rd–70th day of life, frequently resulted in neurological disease including paralysis.[4] Data from the present study revealed that CsA treatment abrogated the normal age-related resistance to JHMV of rats challenged on the tenth, 16th and 35th day of age (Table 1). However, to be effective the drug had to be administered 2 days ahead of the virus inoculation and continually thereafter, as with rats in group 2b of Table 1. When initiation of CsA treatment was delayed until 10th day after injection of virus, as in group 2c, or until the 14th day, as in group 4c, or when treatment around the time of inoculation was terminated and not resumed, as in group 4d, no disease developed presumably because resistance was maintained. Evidently, CsA promoted the maintenance and replication of JHMV in the CNS.

Table 2 Relationship between immunosuppression with CsA and time elapsing before virus activation in the CNS of Wistar Lewis rats

Days post-inoculation[a]	Symptoms	Controls pfu/g brain	Symptoms	Treated with CsA[b] pfu/g brain
1	–	0	–	0
3	–	0	–	0
5	–	0	–	0
15	–	0	+	ND
18	[c]+	3.7×10^3	–	5.5×10^5
21	–	0	+	3.1×10^6
22	–	0	+	1.4×10^5
22	–	0	+	6.9×10^4
24	–	0	+	ND
28	–	0	+	1.0×10^4
30	–	0	+	9.0×10^4
31	–	0	+	ND
35	–	0	+	1.1×10^5
56	–	0	–	0

[a] All rats were inoculated i.c. at 35 days of age with 8×10^5 pfu.

[b] On the 33rd day postpartum daily injections of CsA were commenced, terminated on the 37th day and resumed on the 48th day, then continued until the time of sacrifice.

[c] This single untreated animal developed chronic paralytic disease symptoms, as in Sorensen *et al.*[2]

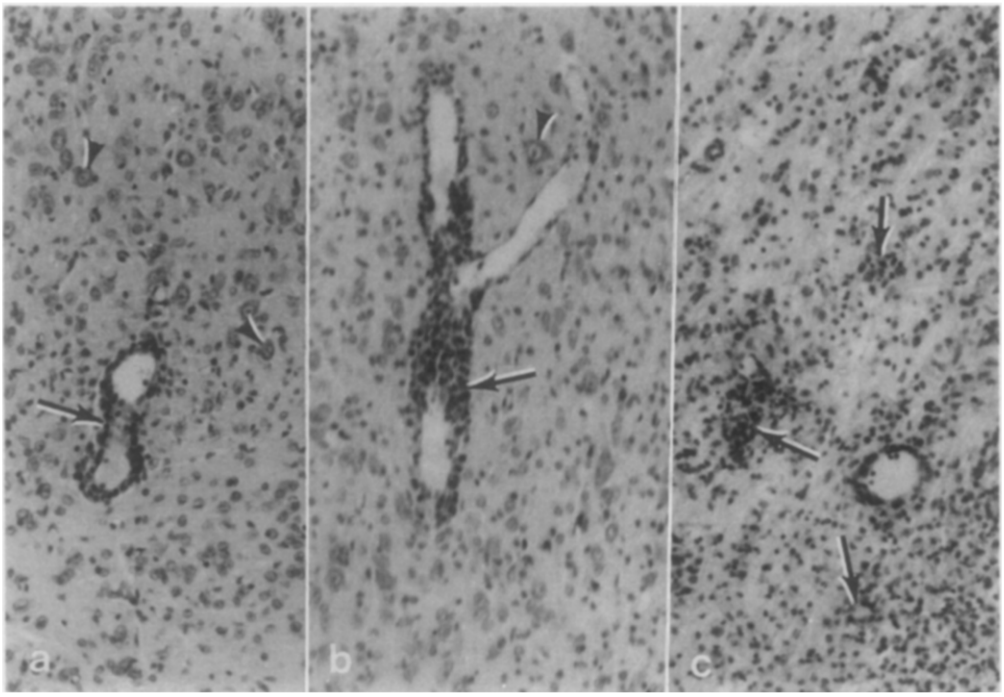

Fig. 1. Inflammatory response in the CNS of a WL rat inoculated with JHMV at 10 days of age and sacrificed 14 days later. In (a) and (b) arrows indicate perivascular cuffing in the myelencephalon; in (c) is shown an area of the spinal cord containing mononuclear infiltrates (arrows) within the neuropile which is normal in appearance. Individual neurons are identified by arrowheads. ×400.

Cyclosporin A treatment and activation of JHMV in the CNS

An association between CsA treatment and manifestation of disease symptoms in post-weanling WL rats led us to carry out a systematic testing for presence of infectious virus. As shown in Table 2, all tested rats manifesting disease symptoms contained high titers of JHMV in their brains. In one case the quantity exceeded 3×10^6 pfu/g. With age-related controls untreated with CsA, the virus was detected in a single rat out of the group of 11 tested. In this animal, in which spontaneous activation had occurred, the titer was 3.7×10^3 pfu/g brain, which is over 2 $\log_{10}$ less than the pfu found in an age-related CsA treated littermate (Table 2).

Efficient clearing and eclipse of JHMV in less than one day after i.c. inoculation was evident from the assays carried out on brains of CsA-treated and control rats (Table 2). This unambiguous result showed that the input virus did not contribute to JHMV titers detected on the 18th day post-inoculation and beyond. The combined data in Tables 1 and 2 indicate that the effects of CsA have to be in place around the time of virus inoculation and continually thereafter (group 2b of Table 1) or must be present within 10 days after the initial treatment (group 4b of Table 1), if a reactivable latent type JHMV infection is to be established and maintained.

Effects of JHMV infection and cyclosporin A on histopathology in the CNS

When WL rats older than ten days are inoculated i.c. with $5\times10^4-10^5$ pfu of JHMV, neurological disease symptoms are very rarely manifested.[3] In the present study we examined histological sections prepared from various CNS regions of rats inoculated on the 10th day. Among group 1a animals of Table 1, there was no evidence of any tissue necrosis. However, prominent inflammatory responses were clearly present.

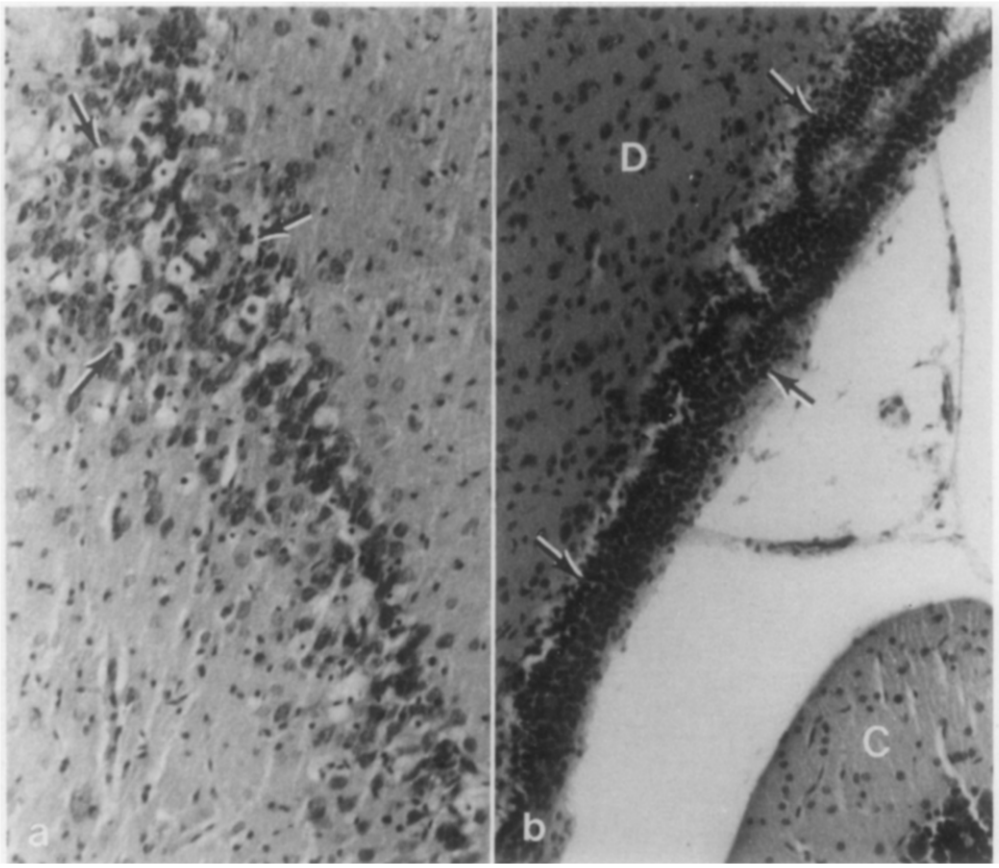

Fig. 2. Regions of the brain of a WL rat which had been infected with JHMV and treated with CsA, as in protocol 2b of Table 1 and killed at 47 days of age. In (a) region of the hippocampus illustrating extensive neuronal destruction (arrows); (b) region of the diencephalon (D), adjacent to the cerebellum (C), where extensive infiltration of mononuclear cells into the meninges is indicated by arrows. ×400.

These responses were in the form of perivascular cuffing [Figs. 1(a) and (b)] and mononuclear cell infiltration into the neuropile [Fig. 1(c)]. Very similar inflammatory responses were found in the CNS of CsA untreated rats in group 2a, infected on the 16th day and in group 4a rats infected on the 35th day, as illustrated in Figs 2(a) and (b). By contrast, multiple lesions were present in the CNS of CsA-treated litter mates in groups 2b and 4b but in these animals the signs of inflammation were absent or much less prominent [Fig. 2(c)]. Presence of lesions could be correlated with disease symptoms as well as high virus titers (data in Table 2). The disease process associated with CsA was manifested rapidly, initially as lethargy or ataxia on the first day and hind leg paresis on the second day. These rats, unless sacrificed in time, invariably died within 48–72 h after appearance of the early symptoms. This rapidly developing disease should be contrasted with the prolonged, chronic, progressive paralysis associated with JHMV infection of untreated animals.

Tissue necrosis in groups 2b and 4b was principally in grey matter tissue of the diencephalon and the mesencephalon [Fig. 3(a)]. In the spinal cord and rhombencephalon, including the cerebellum, both grey and white matter necrotic foci were found frequently. A rarely observed feature of histopathology among CsA treated rats of group 4b were massive mononuclear cell infiltrates at the meninges. One example in the diencephalon region close to the cerebellum is illustrated in Fig. 3(b).

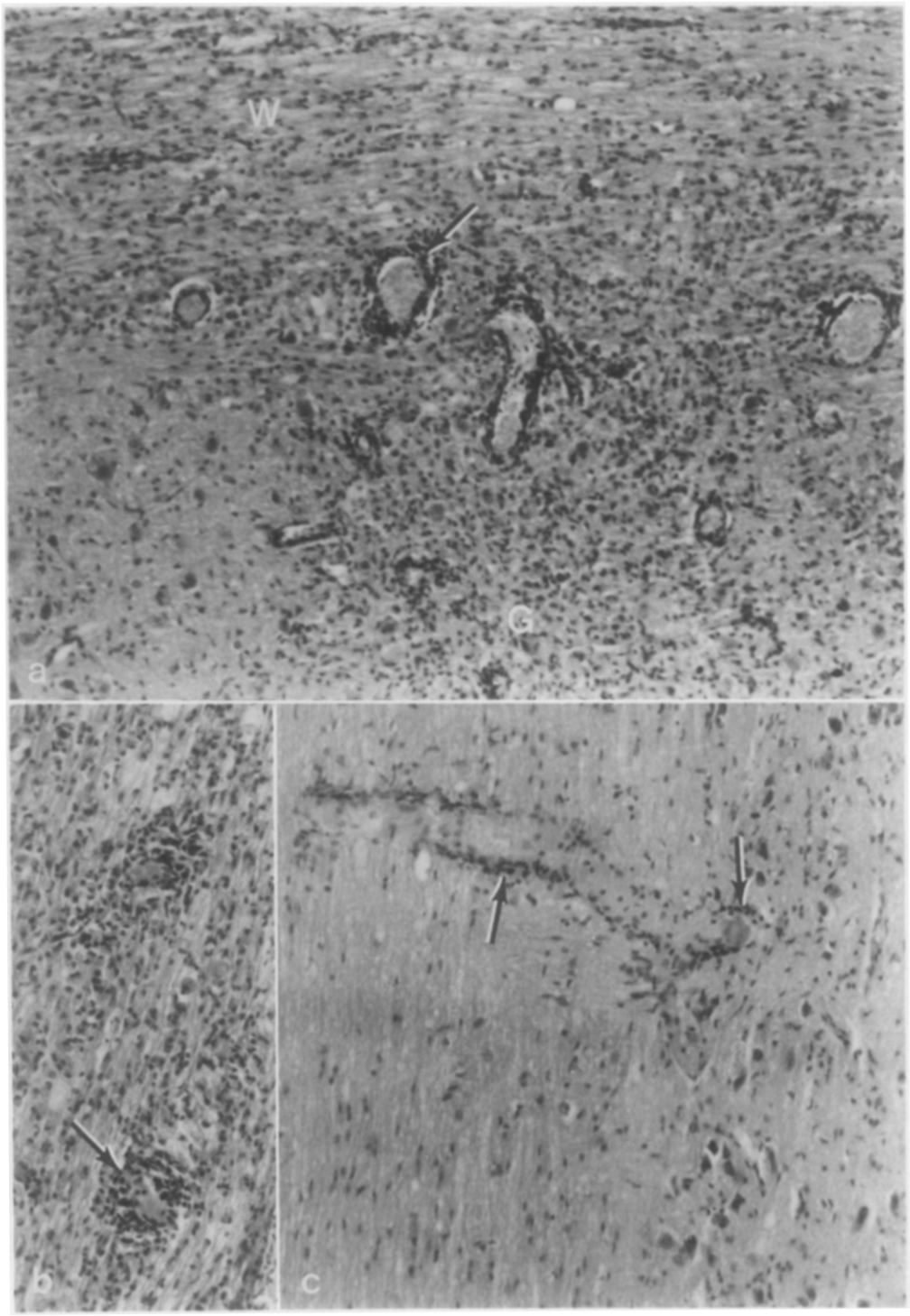

Fig. 3. Examples of regions in the spinal cord from WL rats infected when 35 days old, and either left untreated until sacrificed on the 57th day (a), (b), or immunosuppressed with CsA according to protocol 4b in Table 1, then sacrificed on the 53rd day (c). In (a) there is evidence of necrosis in grey (G) and white (W) matter. In (a) and (b) perivascular cuffing (arrows) and extensive mononuclear infiltrates are evident. In (c) the cuffing and infiltration into the neuropile are sparse. (a) ×260; (b) and (c) ×400.

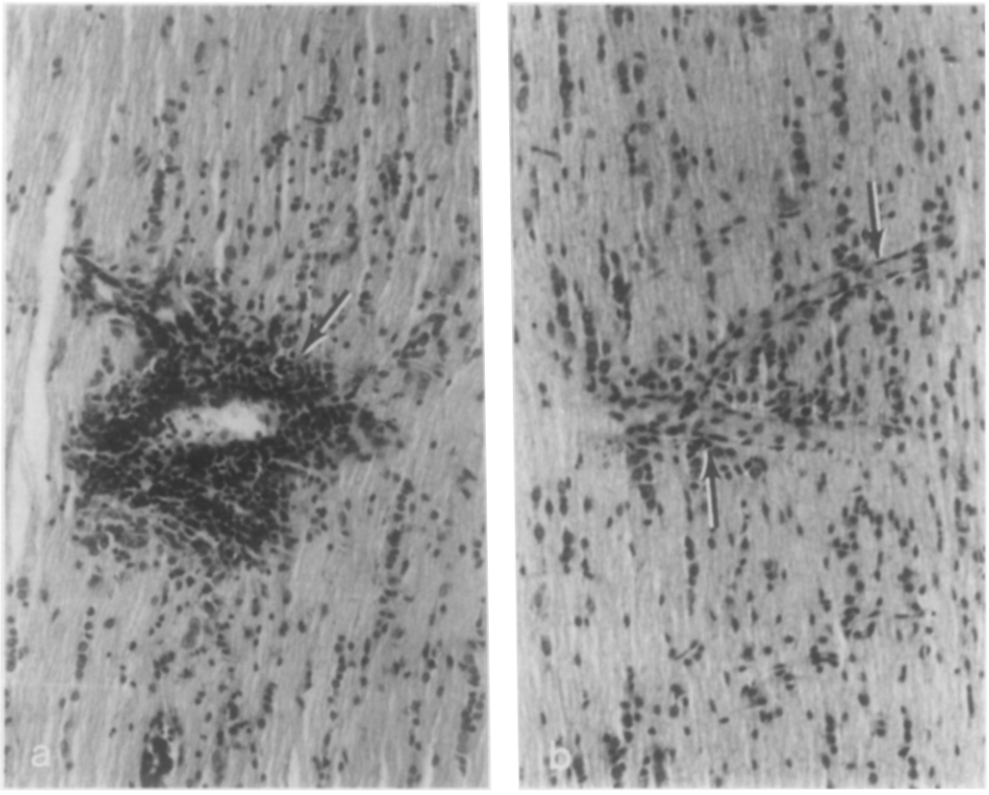

Fig. 4. Comparison of the histological manifestation of an inflammatory response evident in the spinal cord of (a) WL and (b) WF rats, immunized at 50 days of age with guinea pig neural antigens. The animal in (a) was sacrificed at 66 days and in (b) at 62 days of age. Compare the massive perivascular cuffing in (a) with an apparent absence of inflammation in (b). ×400.

Comparison of autoimmune disease induced by neural antigen in WF and WL rats

To determine whether inflammatory responses and CNS necrotic lesions observed after JHMV infection in WL rats were similar to those found during experimental allergic encephalomyelitis (EAE), an autoimmune disease induced by neural antigen(s), we immunized 4–5 week old animals with homogenates of guinea pig spinal cord (GPSC) according to the experimental protocol of Feurer *et al.*[12] All nine WL rats receiving GPSC developed chronic-relapsing EAE exactly as described by Feurer *et al.*[12] Within 10 days of injection of antigen into the hind foot pads. Among the initial symptoms were loss of tail tonicity which gradually became more severe, progressing to a hind leg paresis. Within the next week, these animals recovered but then suffered a relapse within the next two weeks. Histological sections of the spinal cord and cerebellum of animals sacrificed when displaying hind leg paresis revealed extensive perivascular cuffing in the spinal cord [Fig. 4(a)] and a considerable infiltration in the cerebellar tissue.

For comparison, six age-matched WF rats, which manifest only low grade inflammatory processes after JHMV infection,[3] when injected with GPSC, as above, failed to develop any symptoms of EAE. However, within four days of injection some edema and inflammation were observed on the hind feet among this WF group, but, these symptoms disappeared permanently during the subsequent three days. Sections of the CNS surveyed for histopathology failed to reveal tissue destruction or evidence of

inflammation [compare Figs 4(a) and (b)]. From these observations we conclude that WF rats are not susceptible to the type of EAE provoked by neural antigens in WL rats.

Discussion

Aspects of neurological disease of rats induced by JHMV which were considered in the present study are (a) resistance to i.c. infection during postnatal development, and (b) the role of cellular immunity and possible relationship to the hosts' genetic endowment. In WL rats the resistance to JHMV, if limited to an inoculum of 5×10^4–10^5 pfu, sets in by the 10th day of age,[3] but can be surmounted with increasing quantities of virus. As a consequence, even when 35 day old rats are challenged, they occasionally succumb to a late onset, chronic paralytic disease in which demyelination of the white matter predominates.[2] This finding implies that after weaning, when CNS myelination is completed in the rat there is a lower probability of JHMV infecting susceptible oligodendrocyte targets for which this virus has a specific tropism.[9]

Treatment of WL rats with CsA at concentrations known to suppress cellular immunity,[13,14] effectively abrogates the age-related resistance to JHMV, even in 35 day old animals. However, the ensuing disease process is different from that in the untreated controls but is virtually identical to one observed in post weanling athymic, rnu/rnu rats studied by us previously.[15] Among both the nude and CsA treated groups there is only a brief interval of two or three days between appearance of symptoms, onset of paralysis, manifestation of convulsions and death. CNS histopathology of these immunodeficient rats reveals that neuronal infection predominates being most prominent in the hippocampal and rhombocephalic areas where multiple lesions are encountered. The histopathology may explain why the course of the disease is so rapid. Occurrence of necrotic lesions, albeit small, in the white matter, indicates that oligodendrocytes and perhaps other non-neuronal cell types may also be affected. Another striking but rarely encountered similarity between CsA-treated and nude rats is an accumulation at the meninges of massive quantities of mononuclear cells as shown in Sorensen *et al.*,[4] and Fig. 2(b) here.

The different forms of CNS disease which develop in normal versus immunodeficient rats are undoubtedly connected with cellular rather than humoral immunity, because intrathecal production of anti-JHMV immunoglobulin does not appear to influence the progress of the chronic disease in euthymic rats.[11] Spread of infection to neurons may occur readily in CsA treated rats because a reservoir of JHMV is maintained at specific sites of grey matter in the brain, as revealed by *in situ* probing.[15] Presumably, when cellular immunity is lacking the spread of virus from the repository sites cannot be interrupted and the infection becomes disseminated. The rather limited involvement of the white matter in the disease of older CsA treated rats is perhaps due to the paucity of susceptible oligodendrocyte targets, since this cell type becomes refractory to infection by JHMV after the age-related differentiation has occurred.[10]

In this study, we were able to demonstrate a correlation between replication of JHMV in the CNS and manifestation of disease. Among the rats infected at 35 days of age, one control animal which developed a late-onset paralysis and virtually all those treated with CsA contained virus in the brain (Table 2). Undoubtedly, this was progeny virus because the infectiousness of the inoculum is rapidly eclipsed to undetectable levels. However, the infectious potential of the inoculum has to be maintained in the brain for the virus to become subsequently triggered into productive replication, which occurs very often in immunosuppressed rats and rather infrequently in untreated animals. From studies with normal WF rats, there is evidence that latency

with JHMV occasionally can be very prolonged, whereby virus expression in the CNS is detectable beyond 138 days after i.c. inoculation.[11] The initial period of a few days around the time of inoculation must be critical for establishing the latent virus reservoir because immunosuppression with CsA has to be in effect during this decisive interval. Furthermore, to perpetuate the infection CsA treatment must be maintained or resumed, within 10 days, suggesting that in pre-weanling WL rats cellular immunity can effectively keep in check and perhaps entirely eliminate the infection.

Evidence of others that JHMV induces the proliferation of T cells recognizing myelin antigen(s), specifically MBP and that such T cells can be used to adoptively transfer EAE,[16–18] provided suggestive data favouring the idea that demyelination occurs as a consequence of an autoimmune process triggered by the infection. Occurrence of prominent inflammatory responses in the form of perivascular cuffing and monocytic cell infiltrates into the neuropile, reported previously by others,[16–19] and noted here is consistent with the autoimmune mechanism of demyelination. However, the similarity in the paralytic disease, with predominantly white matter demyelination, which develops in WL and WF rats infected with JHMV brings into question the relevance of autoimmunity to the chronic CNS disease of rats because in the WF strain inflammatory responses are either minimal or absent (unpublished observations from this study). It should also be noted from this study that WF rats are not inducible for EAE by immunization with neural antigen(s), providing additional evidence against the likelihood that demyelination associated with JHMV is the consequence of an autoimmune disease process.

From the current findings we continue to favour the hypothesis that in JHMV infected rats, the paralytic form of the disease which develops and is associated with demyelination, is the consequence of an infection of the oligodendrocyte targets. The simultaneous tropism of this virus for neurons can result in an acute encephalomyelitis, evident when very young rats are challenged,[1–3] or in a grey matter disease of the type described here, when older athymic[4] or CsA treated rats are infected.

Materials and methods

Animals. Inbred Wistar Lewis (WL) and Wistar Furth (WF) rats were obtained as breeding stock from Harlan Sprague Dawley and bred in house as reported previously.[3]

Preparations of CNS samples for histopathology. After removal of the CNS from control rats or those manifesting disease symptoms, the tissue was divided sagitally into equal halves. One half was homogenized and employed for estimation of virus titer by assay for plaque forming units (pfu). The other half was fixed in 10% buffered formaldehyde, at neutral pH, dehydrated, embedded, sectioned and stained with hematoxylin and eosin, as described previously.[2]

Virus inoculation and assays. JHMV was propagated on L-2 cells and quantitated by plaque assay as previously described.[15] The pfu of the inoculum injected i.c. was varied, depending on the experimental group involved. As described in the legend of Table 1, rats in groups 1 and 2 received 10^5 pfu at 10 and 16 days of age, respectively. Those in group 3 were injected at 15 days of age with 2×10^5 pfu. Rats in group 4 received 8×10^5 pfu at 35 days of age. Titers of JHMV in the CNS were obtained by plaque assay on L-2 cell monolayers by means of a dilution series of 10% brain homogenates as in Sorensen *et al.*[3] The fate of the infectious inoculum, in the brain was determined at 1, 3 and 5 days post-inoculation.

Immunosuppression with cyclosporin A. Cyclosporin A (CsA), obtained from Sandimmune (Sandoz Canada Inc.), was injected intraperitoneally (i.p.) to deliver 25 mg/kg/day, according to the schedule shown in Table 1.

Chronic-relapsing experimental allergic encephalomyelitis (CREAE). To induce the chronic

relapsing type of EAE, as described by Feurer *et al.*,[12] spinal cords from guinea pigs (GPSC) were isolated from 500–800g animals, homogenized and prepared as an emulsion for inoculation. The GPSC antigen in emulsified complete Freund's adjuvant with BCG cell walls added, was injected into the hind foot pads of 8 week old WL and WF rats. Assessment of EAE was according to criteria of Feurer *et al.*[12]

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