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Short Communication

$CD4^+$ and $CD8^+$ T cells are not major effectors of mouse hepatitis virus A59-induced demyelinating disease

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We examined murine hepatitis virus strain A59 (MHV-A59)-induced demyelinating disease in C57BL/6 mice which had previously been thymectomized at 25 days of age. Demyelination was observed in 51–96% of spinal cord quadrants examined 30 or 60 days post infection (dpi), indicating that neither an intact thymus nor thymic infection is a prerequisite to demyelination. Depletion of $CD4^+$ or $CD8^+$ T cells at 5, 7 or 10 dpi did not influence the extent of demyelination indicating that neither T cell subset is a major effector of demyelination. However, these findings do not exclude the possibility that T cells are involved in initiating demyelinating disease very early in infection.

Keywords: coronavirus; demyelination; T cell; thymectomy

Mice infected intracerebrally with the neurotropic coronavirus MHV-A59 develop a biphasic disease. The acute phase of the disease corresponds to the replication of virus in various organs and is manifested as a sometimes lethal hepatitis and encephalomyelitis. Mice recovering from acute disease progress to a chronic demyelinating disease which resembles human multiple sclerosis in the development of primary demyelinating lesions confined to the central nervous system, and accompanying severe neurological symptoms. Hence, the elucidation of the mechanisms leading to MHV-induced demyelination in this model may ultimately contribute to an understanding of and successive intervention in multiple sclerosis.

The detection of viral particles in oligodendrocytes led to the theory that MHV-induced demyelination was directly due to viral damage of these cells (Lampert *et al*, 1973). However, more recently it has been shown that immunosuppression, by irradiation of infected mice, prevented demyelination, despite a corresponding increase in viral titer (Wang *et al*, 1990; Fleming *et al*, 1993). This, together with the finding that demyelination was restored by the adoptive transfer of $Thy1^+$ splenocytes (Fleming *et al*, 1993) suggested that T cells may be mediators of demyelination. In contrast to these studies, we have shown that $CD8^+$ T cell deficient $\beta 2M^{-/-}$ gene knockout mice undergo demyelination, indicating that $CD8^+$ T cells are not absolutely necessary for demyelination (Gombold *et al*, 1995). However, the frequency at which demyelination was observed in these animals was very low, a result which may be attributed to either a role for $CD8^+$ T cells in demyelination in normal mice, or an inefficient infection rate due to the use of a low inoculating dose of wild type MHV-A59 in $\beta 2M^{-/-}$ knockout mice (LD_{50}=5 pfu). While infection could reliably be established using C12, an attenuated variant of A59 (LD_{50}=200 pfu in knockout mice), the question of the relative importance of $CD8^+$ T cells in demyelinating disease remained in some doubt as C12 induces demyelinating lesions at very low frequency in both wild type and $\beta 2M^{-/-}$ knockout mice. Hence, in the present study we have attempted to resolve this question by carrying out *in vivo* depletions of $CD8^+$ T cells in normal C57BL/6 mice injected with one LD_{50} (3000 pfu) of wild type MHV-A59. Similarly we have extended our earlier study by addressing the role of $CD4^+$ T cells.

C57BL/6 mice were obtained from Taconic Farms (Germantown, NY, USA) at 30–33 days of age and injected intracerebrally with 3000 pfu of MHV-A59 on the day following delivery. Mice had been

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thymectomized by the supplier at 25 days of age, a procedure which facilitated the development of a pool of mature circulating $CD4^+$ and $CD8^+$ T cells, while preventing a continual output of $CD4^+$ and $CD8^+$ T cells from the thymus. This enabled us to maintain the depletion of $CD4^+$ or $CD8^+$ T cells by the administration of a short course of appropriate antibody (three or four intraperitoneal injections of 0.5 mg of protein G purified anti-CD4 antibody GK1.5 or anti-CD8 antibody 2.43 given on consecutive days according to the protocol described by Kruisbeek, 1994). Importantly, mice in which thymic remnants were detected at the time of euthanasia were excluded from the data sets reported here.

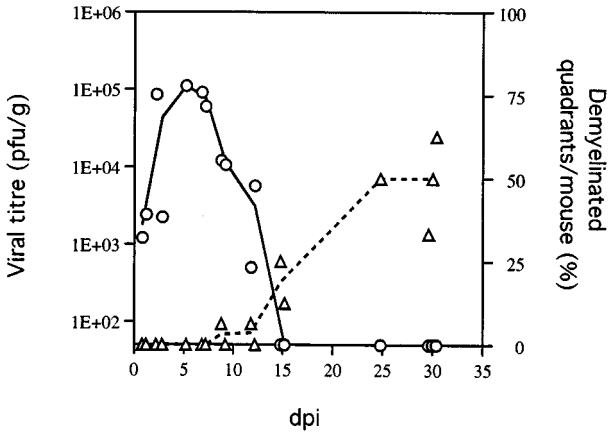

Figure 1 MHV-A59 viral clearance and demyelination in the spinal cord of C57BL/6 mice previously thymectomized at 25 days of age. Both average viral titers (solid line) and titers measured in individual mice (circles) are shown. The limit of detection for the virus plaque forming assay performed on L2 cells was approximately 70 pfu/g of tissue. Demyelination was calculated as the percentage of spinal cord quadrants containing lesions, and both average (broken line) and individual (triangles) scores are shown.

Initially we showed that the course of disease in thymectomized mice (not treated with antibody) was similar to that previously observed in normal mice. Mice were selected randomly at various times post infection and perfused with phosphate buffered saline. The spinal cord from cervical through lumbar regions was removed, and then cut into approximately 3 mm lengths. Alternating lengths were either homogenized and assayed for infectious virus, or formalin fixed and stained with luxol fast blue for detection of demyelinating lesions. During the first week virus titers in the spinal cord rose rapidly, peaking around 3–7 dpi at approximately 10^5 pfu/g. Virus was subsequently cleared and replicating virus was not detectable by 15 dpi (Figure 1). Similar kinetics were observed in the brain, while virus was cleared more rapidly from the liver (data not shown). Spinal cord demyelination was first detected at 9 dpi when virus titers were still high, but continued to increase after replicating virus was no longer detected. By day 30 all mice, and 142/220 (65%) of quadrants, examined exhibited lesions (three mice in Figure 1 and ten mice in the 'no treatment' groups in Table 1). Demyelination was first detected in the brain at 15 dpi when infectious virus was no longer detected (data not shown). Physically mice became hunched and ruffled during the first few dpi, with a progression to waddling gait and partial hind limb paralysis. Indeed we have noted gait abnormalities in such mice as long as 60 dpi.

The observation of demyelination in thymectomized mice is of interest given that we have previously shown that thymus pathology (cortical depletion of lymphocytes) always accompanied MHV-A59 acute infection in C57BL/6 mice (Lavi *et al*, 1990). In addition, autoreactive T cells have been derived from MHV-JHM infected rats (Watanabe *et al*, 1983) and mice (Kyuwa *et al*, 1991) and may contribute to demyelination. Kyuwa *et al* (1991) have speculated that MHV infection of thymic

Table 1 Effect of $CD4^+$ or $CD8^+$ T cell depletion on the incidence of MHV induced demyelination examined at 30 days post infection

Day of commencement of antibody treatment	In vivo *antibody treatment*	n^a	*Spleen cells (%)* $CD4^+$	$CD8^+$	*Demyelination* Score[b]	(%)
7 dpi	None (PBS)	4	8.0±1.1	3.7±0.5[c]	50/64	78
	anti-CD4 (GK1.5)[d]	8	2.9±0.8	3.1±0.9	73/116	63
	anti-CD8 (2.43)	9	8.6±3.0	1.0±0.5	89/140	64
10 dpi	None (PBS)	6	8.0±1.0	4.2±2.6[e]	70/112	63
	anti-CD4 (GK1.5)	6	0.3±0.7	5.7±2.4	57/111	51
	anti-CD8 (2.43)	6	10.4±2.9	0.0±0.0	79/112	71
	anti-CD4 and CD8 (GK1.5 and 2.43)	5	0.7±0.8	0.0±0.0	46/90	51

[a] Number of mice examined
[b] Number of spinal cord quadrants exhibiting demyelination/number of quadrants examined
[c] Direct immunofluorescent staining assessed using a Becton-Dickinson FACScan for spleen cells 7 dpi
[d] Four immunizations of 0.5 mg of antibody was administered to this group of mice on consecutive days; three immunizations were used for all other depletions
[e] Indirect immunofluorescent staining assessed using a Becton-Dickinson FACScan for spleen cells 10 dpi

epithelium may interfere with thymic negative selection resulting in the escape to the periphery of such autoreactive T cells. While the present study does not directly address the role of autoreactive T cells in demyelinating disease, it does clearly show that if such cells are important, they do not arise as a consequence of thymic infection.

We next examined the effect of $CD4^+$ or $CD8^+$ T cell depletion at various time points post infection on the incidence of demyelination. An attempt was made to examine the role of $CD4^+$ or $CD8^+$ T cells in demyelination in the early stages of infection by MHV-A59 by commencing the depletion of each of these subsets on 5 dpi. Unfortunately, of six mice in each group infected in this experiment all those depleted of $CD8^+$ cells and four depleted of $CD4^+$ cells died 15 dpi. This result was unsurprising given that viral titers are at a peak at 5 dpi (Figure 1) and both $CD4^+$ and $CD8^+$ T cells are known to be important in MHV clearance (Williamson and Stohlman, 1990; Pearce *et al*, 1994). Indeed, previous attempts to infect immunosuppressed C57BL/6 mice at times earlier than 7 dpi resulted in lethal infections (Lavi and Weiss, unpublished observations). Nevertheless, spinal cords were collected at 30 dpi from the two surviving mice in the $CD4^+$ T cell depleted group, fixed in 4% glutaraldehyde in PBS and examined by toluidine blue staining for demyelinating lesions. Ninety-six percent of spinal cord quadrants examined in these mice (25 out of 26) exhibited demyelination.

In order to reduce the risk of fatalities, this experiment was repeated but the commencement of T cell depletions was delayed until 7 dpi, when viral titers were beginning to decline in thymectomized mice (Figure 1). Spinal cords were collected at 30 dpi and examined by toluidine blue staining for demyelinating lesions as described above. All mice examined in both control and $CD4^+$ or $CD8^+$ T cell depleted groups contained demyelinating lesions; the summed scores for the respective groups are shown in Table 1.

We also performed the experiment beginning depletion of T cells at day 10 when viral titers are waning. This enabled us to also examine the effects of depleting both $CD4^+$ and $CD8^+$ T cells subsets, together, in addition to $CD4^+$ and $CD8^+$ T cells, separately, without risk of persistent viral infection (see Table 1). Indeed, infectious virus was examined at 30 dpi in a random sampling of mice in all the groups shown in Table 1, and was not detected by plaque forming assay. Again, we saw no significant difference between any of the groups of mice with regard to demyelination at 30 dpi, compared to the control group, including the five mice in which both $CD4^+$ and $CD8^+$ T cells were depleted (Table 1). A statistical comparison of scores for individual animals by the Mann-Whitney U test indicated that the scores for T cell depleted groups in Table 1, did not differ significantly from the control undepleted mice ($P>0.05$).

Finally, in order to exclude the possibility that $CD4^+$ or $CD8^+$ T cells were important effectors of demyelination at later stages post infection, mice were depleted of $CD4^+$, $CD8^+$ or both $CD4^+$ and $CD8^+$ T cells, commencing at day 10 and examined for demyelination at 60 dpi with the results shown in Table 2. It is of interest that the groups which had received *in vivo* T cell depletion show an increase in demyelination compared to the control group. This appears to be because some lesions had healed in the undepleted mice which suggests that T cell depletion may have decreased the rate of remyelination. However, these differences were not significant by a Mann-Whitney U test ($P>0.05$) and further studies would be required to directly confirm this possibility. Nevertheless, it is clear that demyelination is neither reduced nor delayed in T cell depleted mice.

In the experiments shown in Tables 1 and 2, a total of 18 mice were depleted of $CD4^+$ T cells, 18 were depleted of $CD8^+$ T cells and ten were depleted of both T cell subsets. The degree to which T cell depletion was maintained during the experiment was assessed at the end of the experiment, at 30 dpi or 60 dpi, and was found to be successful ($<1\%$) for a majority but not all animals. We thus examined whether there was any statistical correlation between residual numbers of T cells and the degree of demyelination for individual animals and found none ($r=0.08$ for $CD4^+$ T cell depleted mice and $r=-0.08$ for $CD8^+$ T cell depleted mice).

Table 2 Effect of $CD4^+$ or $CD8^+$ T cell depletion, commenced at day 10, on the incidence of MHV induced demyelination examined at 60 days post infection

In vivo *antibody treatment*	n^a	*Spleen cells (%)*[b]		*Demyelination*	
		CD4$^+$	*CD8*$^+$	*Score*[c]	*(%)*
None (PBS)	5	8.5 ± 1.3	5.8 ± 1.0	33/96	34
anti-CD4 (GK1.5)	4	1.5 ± 0.5	7.6 ± 0.9	49/74	66
anti CD8 (2.43)	3	10.0 ± 0.5	0.1 ± 0.2	32/52	62
anti-CD4 and CD8 (GK1.5 and 2.43)	5	3.0 ± 1.5	0.0 ± 0.0	61/94	65

[a] Number of mice examined
[b] Indirect immunofluorescent staining assessed using a Becton-Dickinson FACScan
[c] Number of spinal cord quadrants exhibiting demyelination/number of quadrants examined

Altogether, these results suggest that neither $CD4^+$ nor $CD8^+$ T cells are the major effectors of MHV induced demyelinating disease, but do not exclude a minor role of these T cells as effectors of demyelination. Furthermore, this study does not address the role of $CD4^+$ and $CD8^+$ T cells in the initiation of demyelinating disease very early in infection. Given our results, and those of Stohlman and colleagues (Wang *et al*, 1990; Fleming *et al*, 1993) which show that demyelination is reduced only if mice are immunosuppressed by irradiation at 6 dpi or earlier, with restoration of demyelination upon adoptive transfer of $Thy1^+$ splenocytes, it seems probable that T cells may play a role in induction of demyelinating disease rather than as effectors. It is possible that a component of early demyelination, prior to clearance of infectious virus, may be due to direct virus mediated lysis of oligodendrocytes. However, this seems unlikely to be responsible for the majority of the demyelination which arises when infectious virus is no longer detected. The failure to suppress demyelination via irradiation at 7 dpi or later (Wang *et al*, 1990) would be consistent with a role for more radiation resistant effectors such as macrophages dependent on activation via T cells in the initial stages of the disease. In this regard, although $CD4^+$ T cells play a critical role in demyelination in experimental autoimmune encephalomyelitis and an as yet undefined role in multiple sclerosis (reviewed by Martin *et al*, 1992), macrophages are also conspicuous in the inflammatory lesions of these demyelinating diseases (Prineas and Connell, 1978; Epstein *et al*, 1983; Traugott, 1985).

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