

Rapid Entry and Downregulation of T Cells In the Central Nervous System During the Reinduction of Experimental Autoimmune Encephalomyelitis

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Abstract

We investigated the mechanisms whereby a previous attack of experimental autoimmune encephalomyelitis (EAE) modifies a subsequent attack in the Lewis rat. Active immunization with myelin basic protein (MBP) and complete Freund's adjuvant 28 days after the passive transfer of MBP-sensitized spleen cells induced a second episode of EAE, which occurred earlier than in naive control animals, but was less severe overall. The pattern of neurological signs was also different in rechallenged rats, which had less severe tail and hindlimb weakness but more severe forelimb weakness. In rechallenged rats, inflammation was more severe in the cervical spinal cord, cerebellum, brainstem and cerebrum, but less severe in the lumbar spinal cord, than in controls. The early onset of EAE in rechallenged rats was explained by a memory T cell response to MBP₇₂₋₈₉ in the draining lymph node and spleen, and by the enhanced entry of T cells into the central nervous system (CNS). However, the number of $\alpha\beta$ T cells in the spinal cord of rechallenged rats declined faster than in controls, especially in the lumbosacral cord, where the number of V β 8.2⁺ T cells and the frequency of T cells reactive to MBP₇₂₋₈₉ rapidly decreased, indicating rapid downregulation of the immune response in the previously inflamed spinal cord. Apoptosis of inflammatory cells in the CNS was increased in the rechallenged rats and is likely to contribute to this downregulation. Furthermore, during the disease course the generation of encephalitogenic T cells in the peripheral lymphoid organs was limited compared with controls. Thus, a previous attack of EAE modifies a subsequent attack through the interaction of the following processes: a memory T cell response to MBP; facilitated T cell entry into the CNS; downregulation of the immune response in the CNS, including increased apoptosis of inflammatory cells; and a limited generation of encephalitogenic T cells in the peripheral lymphoid organs.

Keywords: apoptosis; experimental autoimmune encephalomyelitis; memory; T cell; tolerance

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a T-cell-mediated autoimmune demyelinating disease of the central nervous system (CNS) that serves as an animal model of the human inflammatory demyelinating disease, multiple sclerosis (Pender, 1995). Acute EAE can be induced in the Lewis rat by active immunization with myelin basic protein (MBP) and complete Freund's adjuvant, or by the passive transfer of MBP-sensitized lymphocytes. The rats develop stereotyped neurological signs with a predictable temporal profile: ascending tail paralysis followed by hindlimb weakness and occasionally forelimb weakness (Pender, 1986). They rapidly and spontaneously recover so that by about 7 days after the onset of neurological signs there is no residual weakness. The process of spontaneous recovery is not yet fully understood and many factors have been postulated to play a role, including endogenous corticosteroids (Levine et al., 1980; MacPhee et al., 1989), suppressor cells (Swierkosz and Swanborg, 1975; Welch et al., 1980) and nitric oxide (O'Brien et al., 1999). Furthermore, during clinical recovery there is apoptosis of inflammatory cells in the CNS (Pender et al., 1991), which involves T cells (Pender et al., 1992; Schmied et al., 1993), B cells (White et al., 2000), macrophages (Nguyen et al., 1994; White et al., 1998a) and microglia (White et al., 1998a) and

contributes to the resolution of inflammation in the CNS. Within the T cell population in the CNS, the apoptotic process selectively eliminates V β 8.2⁺ MBP₇₂₋₈₉-reactive T cells (Tabi et al., 1994, 1995; McCombe et al., 1996), which constitute the predominant encephalitogenic MBP-reactive T cell population in the Lewis rat (Imrich et al., 1995).

After recovery from an episode of actively induced EAE, Lewis rats have generally been found to be resistant to the active reinduction of disease (Willenborg, 1979; Levine and Sowinski, 1980; Hinrichs et al., 1981) and either resistant (Levine and Sowinski, 1980) or susceptible (Willenborg, 1979; Hinrichs et al., 1981) to passively induced EAE. Rats that have recovered from passively induced EAE have been found by some authors to be fully susceptible to the active or passive reinduction of disease (Hinrichs et al., 1981) and by others to be resistant to reinduction by active (Welch et al., 1980; Namikawa et al., 1986) or passive means (Levine et al., 1967). Various mechanisms have been postulated to account for the resistance to reinduction of EAE, especially suppressor cells (Swierkosz and Swanborg, 1975; Welch et al., 1980; and Sun et al., 1988), but little attention has been given to the role of downregulatory factors within the target organ, the CNS, in the rechallenged animals. Target organ resistance has been demonstrated after recovery from another autoimmune disease, experimental autoimmune oophoritis (Lou et al., 1995), and appears to be responsible for the resistance to induction of even the first episode of EAE in AO rats (Mostarica-Stojkovic et al., 1992). Histological examination of the CNS during a mild or subclinical second attack of EAE induced in convalescent Lewis rats has revealed that inflammation is decreased in the spinal cord but enhanced in the cerebellum after rechallenge (Levine et al., 1967; Levine and Sowinski, 1980). The authors concluded that alterations in the distribution of CNS lesions could be explained, not by systemic factors, but by local factors within the target organ.

In the present study, we immunized Lewis rats with MBP in complete Freund's adjuvant after they had recovered from an episode of passively induced EAE and thereby induced a second episode of EAE, which was less severe overall and occurred earlier than in actively immunized naive control animals. Although tail and hindlimb weakness was less severe, forelimb weakness was more severe in the rechallenged than the control rats. We hypothesized that changes within the CNS, induced by the first episode of EAE, might explain this redistribution of neurological signs and the overall milder clinical course. To investigate this, we utilized two-colour flow cytometry, histological studies and limiting dilution analysis to compare the inflammatory process in the lumbosacral spinal cord and cervicothoracic cord in actively induced EAE in naive rats and in rechallenged rats. Our results indicate that the outcome of active rechallenge in convalescent Lewis rats is determined by the interaction of the following mechanisms: a memory T cell response to MBP; facilitated T cell entry into the CNS; downregulatory mechanisms in the CNS, including increased apoptosis of inflammatory cells; and a limited generation of encephalitogenic T cells in the peripheral lymphoid organs.

2. Materials and methods

2.1. Animals

Lewis rats (JC strain), 7–10 weeks old, were obtained from the Central Animal Breeding House of the University of Queensland.

2.2. Antigens

Whole myelin basic protein (MBP) was prepared from guinea-pig brains by the method of Deibler et al. (1972). The major encephalitogenic region of guinea-pig MBP in the Lewis rat, MBP peptide 72–89 (PQKSQRSQDENPVVHF) (Offner et al., 1987), and the encephalitogenic rat MBP peptide 87–99 (VHFFKNIVTPRTP) (Offner et al., 1989) were synthesized by Auspep Pty. Ltd. (Australia). Concanavalin A was obtained from Sigma Chemical Co. (USA).

2.3. Preparation of MBP inoculum and immunization of rats

MBP in saline was emulsified in an equal volume of incomplete Freund's adjuvant containing 4 mg/ml *Mycobacterium butyricum*. Under anaesthesia, rats were inoculated in the left hind footpad with 0.1 ml emulsion. The total dose of MBP was 50 μ g per rat.

2.4. Passive transfer of EAE

Cells for passive transfer were generated according to the method of Panitch and McFarlin (1977). Single-cell suspensions were prepared from the spleens of male or female rats sensitized 10 days previously with MBP as described above. Cells were cultured at a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 5% fetal calf serum (FCS) (Gibco BRL, USA), 5×10^{-5} M 2-mercaptoethanol (Sigma), 216 mg/l L-glutamine (Trace Biosciences Pty. Ltd., Australia), 100 IU/ml penicillin (Trace Biosciences), 100 µg/ml streptomycin (Trace Biosciences) and 10 mM HEPES buffer (Trace Biosciences). Concanavalin A was added at 2 µg/ml, and 50–100 ml cultures were incubated at 37°C in an atmosphere of 5% CO₂–95% air. Cells were harvested after 72 h and washed with saline. Passive EAE was induced in 7-week-old male Lewis rats by the injection of $100\text{--}150 \times 10^6$ viable cells in the lateral tail vein. Age-matched male control rats received 0.5 ml of saline in the lateral tail vein. Neurological signs commenced 3–4 days after passive transfer and consisted of tail and hindlimb weakness. The peak of clinical signs occurred 6 days after passive transfer, and all rats had completely recovered by 12 days after transfer.

2.5. Induction of active EAE

Twenty-five to 30 days after the passive transfer of encephalitogenic splenocytes or an injection of saline, all rats were actively immunized with MBP inoculum as described in Section 2.3. The rats were monitored for clinical signs and used for flow cytometry, histology or limiting dilution assays as described below.

2.6. Clinical assessment of animals

Tail, hindlimb and forelimb weakness were each graded on a scale of 0 (no weakness) to 4 (total paralysis) as previously described (Pender, 1986). The total clinical score was obtained by adding these three scores (maximum=12).

2.7. Histological studies

For both actively immunized naive control and rechallenged groups, one rat was examined histologically on the first day, three on the second day, and one on the third day of neurological signs. Under anaesthesia, the rats were perfused via the aorta with saline followed by modified Karnovsky's fixative (2% paraformaldehyde–2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3–7.4). Five segments of the spinal cord (fifth cervical [C5], sixth thoracic [T6], fourth lumbar [L4] and second and fourth sacral [S2, S4]) were removed, processed and embedded in epoxy resin. Semithin sections (0.5 mm) were stained with toluidine blue. The cerebrum (at the level of the optic chiasm), brainstem and cerebellum were embedded in HistoResin (Reichert-Jung, Germany), sectioned (1 µm), and stained with cresyl fast violet, as previously described (Nguyen and Pender, 1989). Apoptotic cells were quantified by light microscopy with a 100× oil-immersion objective and were recognized by the criteria of Kerr et al. (1994). Levels of inflammation in the CNS were scored based on an estimation of the average number of inflammatory cells per field from more than 20 observed fields of the 40× objective: level 0=no cells; level 1=1–5; level 2=6–10; level 3=11–20; level 4=21–40; level 5=41–60; level 6=61–80; level 7=81–100; level 8=101–150; level 9=151–200; level 10>200 cells per field. The assessor was unaware of the clinical findings and of whether the specimen was derived from control or rechallenged rats. Although involvement of the spinal roots in the peripheral nervous system contributes to the neurological signs of MBP–EAE (Pender et al., 1995), we focused our attention in this study on the CNS involvement because it is difficult to extract sufficient inflammatory cells from the spinal roots to perform flow cytometry and limiting dilution assays.

2.8. Isolation of T cells from the spinal cord and lymphoid organs

After active immunization, groups of control or rechallenged rats were sacrificed and T cells from the spinal cords, draining popliteal lymph nodes and spleens were isolated and analyzed using flow cytometry and limiting dilution assays as described below. Spinal cord inflammatory cells were extracted as previously described (Tabi et al., 1994), except that in this study the vertebral column was split between the T7 and T8 vertebrae into rostral and caudal regions. Single-cell suspensions were prepared from the draining left popliteal lymph node and spleen by teasing apart the tissues and passing

them through a fine nylon mesh. Erythrocytes were removed from the spleen cell suspension using Ficoll-paque (Pharmacia LKB Biotechnology, Sweden). To enrich for T lymphocytes, the spinal cord, spleen and lymph node cells were passed through a nylon wool column, as previously described (Tabi et al., 1994).

2.9. Phenotypic and DNA analysis by flow cytometry

Mouse monoclonal antibodies specific for the $\alpha\beta$ T cell receptor (TCR) (R73) were obtained from Dr J.D. Sedgwick (DNAX Research Institute, Palo Alto, CA, USA). Mouse monoclonal antibody to V β 8.2 (R78) was provided by Dr T. Hünig (Torres-Nagel et al., 1993). Mouse polyclonal IgG1 κ (Dako, USA) was used as a control antibody. The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated sheep F(ab')₂ anti-mouse IgG (Sigma). The primary and secondary antibodies were diluted in phosphate-buffered saline (PBS)-azide (1% FCS–1% sodium azide in PBS) plus 10% autologous rat serum. Cells were first washed with a 1:1 solution of FCS in PBS containing 0.1% sodium azide and then incubated with the primary antibodies for 30 min at 4°C. Cells were then washed, incubated with the secondary antibody for 30 min at 4°C in the dark, and washed twice with PBS. Samples were resuspended in 1 ml ice-cold 50% ethanol and fixed overnight at 4°C in the dark. The ethanol was removed by washing the cells with PBS, and the cells were resuspended in 100–300 μ l of propidium iodide (PI)-staining solution which was freshly prepared by diluting stock solution (5 mg/ml RNase–250 μ g/ml PI in 0.01 M PBS containing 0.1 mM EDTA, pH 7.4) 1:4 with PBS containing 0.1% sodium azide. Samples were analyzed using a FACScan or FACSCalibur and CellQuest software (Becton Dickinson, USA), as previously described (White et al., 1998b).

Using two-colour flow cytometry we analyzed T-cell-enriched cell populations isolated from the spleens, draining popliteal lymph nodes and lumbosacral and cervicothoracic spinal cords of groups of control rats 11–14 days after active immunization and of groups of rechallenged rats 6–9 days after active immunization, and determined the proportions of TCR $\alpha\beta$ ⁺, V β 8.2⁺ and apoptotic cells. Each group consisted of 4–6 rats, and results were obtained from 3 to 5 groups on each of the days studied, except for day 13 for controls when results were obtained from only one group of six rats.

2.10. Limiting dilution analysis

We used limiting dilution analysis (Tabi et al., 1995) to determine the frequencies of T cells reactive to MBP, MBP_{72–89} and MBP_{87–99} in the lumbosacral spinal cords, cervicothoracic spinal cords, draining popliteal lymph nodes and spleens of control and rechallenged rats. Cells were prepared as described under Section 2.8. The culture medium used for this assay was RPMI 1640 supplemented with 216 mg/l L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 0.1 mM sodium pyruvate, non-essential amino acids, 36 mg/l L-asparagine, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES buffer, 5 μ g/ml fungizone and 5% heat-inactivated FCS. Twenty-four replicates of increasing numbers of responder T cells (4–6 dilution steps) prepared from the spinal cord, draining lymph node or spleen, and 5×10^5 irradiated normal rat thymocytes were incubated with 15 μ g/ml MBP, 10 μ g/ml MBP_{72–89} or 10 μ g/ml MBP_{87–99} in 96-well U-bottomed tissue culture trays. All wells were supplemented with 10 U/ml recombinant human interleukin-2 (IL-2) (TECIN™, kindly provided by Hoffmann-La Roche Inc., USA) 24 h after the commencement of the assay, and incubated for 7 days in 5% CO₂–95% air at 37°C. [³H]thymidine (0.5 μ Ci) was added to the wells for the last 12 h of culture. The cells were harvested and assayed for incorporated radioactivity. Stimulated wells were scored as positive if the thymidine incorporation was more than twice the mean background level (dilutions of T cells incubated with thymocytes in the absence of antigens), i.e. when the stimulation index was >2.0 . The frequencies of specifically responding cells were determined as previously described (Tabi et al., 1995). The limit of sensitivity of the assay was 6.7×10^{-6} (1/150000).

Limiting dilution analysis was used to determine precursor frequencies in T-cell-enriched populations isolated from the spleens and draining popliteal lymph nodes of control rats 5 and 9 days after active immunization, and rechallenged rats 5 days after active immunization. These time points were chosen so that we could compare the peripheral lymphoid organ T cell responses in naive and rechallenged rats at the same time after active immunization and just before the onset of neurological signs. In a second set of experiments, limiting dilution analysis was used to determine precursor frequencies in T cell populations from the lumbosacral spinal cords, cervicothoracic spinal cords, spleens and draining popliteal lymph nodes of control rats 11 and 13 days after immunization and of rechallenged rats 6 and 8 days after immunization.

2.11. Statistical analysis

Statistical analysis was performed using either a homoscedastic *t*-test or a heteroscedastic *t*-test, depending on the result of the *F*-test.

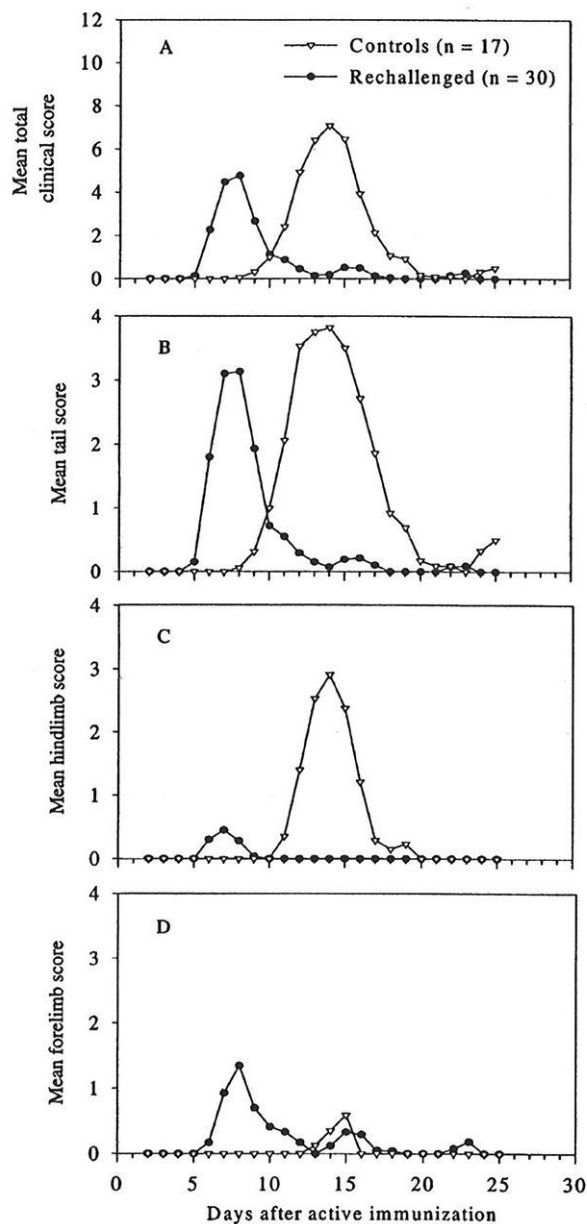


Fig. 1. Mean total clinical scores (A) and mean clinical scores for the tail (B), hindlimbs (C) and forelimbs (D) of rechallenge and control rats after immunization with MBP and complete Freund's adjuvant. The maximal mean total clinical score of rechallenge rats on day 8 was significantly different from that of control rats on day 14 after immunization ($P=0.0006$). Maximal mean tail score of rechallenge rats on day 8 vs. control rats on day 14, $P=0.0005$; maximal mean hindlimb score of rechallenge rats on day 7 vs. control rats on day 14, $P=6.7 \times 10^{-7}$; maximal mean forelimb score of rechallenge rats on day 8 vs. control rats on day 15, $P=0.097$.

3. Results

3.1. Clinical findings

As shown in Fig. 1, rechallenge animals developed EAE earlier than controls. The mean day of onset of neurological signs for rechallenge rats was 6.1 days after active immunization compared with 10.2 in controls ($P=4.1 \times 10^{-11}$). The maximal mean total clinical score (peak of neurological signs) occurred

8 days after immunization (day 8) in rechallenged rats and on day 14 in controls. However, rechallenged rats were relatively resistant to this second attack of EAE. The mean total clinical score at the peak of disease for rechallenged rats was significantly lower than that of control animals (Fig. 1A). Clinical signs in control rats were typical of an episode of acute EAE: ascending paralysis, severely affecting the tail and hindlimbs but only occasionally involving the forelimbs. However, neurological signs in rechallenged rats did not follow a typical ascending course but predominantly involved the tail and forelimbs, with the hindlimbs often totally unaffected. In rechallenged rats the severity of tail weakness, and more particularly of hindlimb weakness, was significantly less than in controls (Fig. 1B, C), whereas the forelimbs were more severely affected, although the difference was not statistically significant (Fig. 1D). Occasionally, rechallenged rats developed a head tilt, emphasizing the rostral redistribution of neurological signs.

3.2. Histological findings

Histological studies demonstrated that, early in the clinical course, inflammation was more severe in the cervical spinal cord than in the lumbar spinal cord in rechallenged rats, whereas in control rats the lumbar cord was more severely affected (Table 1 and Fig. 2 A–D). Furthermore, even on the first day of neurological signs in rechallenged rats, lesions were detected in the cervical spinal cord, cerebellum (Fig. 3B), brainstem and cerebrum as well as in the lumbar spinal cord (Table 1). In contrast, during the first 2 days of neurological signs in controls, there was minimal inflammation in the cervical spinal cord and brainstem and no inflammation in the cerebrum and cerebellum (Table 1 and Fig. 3A). There were more apoptotic cells in the parenchyma of the lumbosacral cord in rechallenged rats (Fig. 3C) than in controls, despite the fact that there were fewer inflammatory cells (Table 1). Histological studies of rats with passively transferred EAE, without active rechallenge, demonstrated that there was no residual inflammation in the spinal cord or brain 35 days after transfer, at a time when the rechallenged rats showed prominent inflammation in the CNS.

TABLE 1. Histological findings

Group of rats	Day of neurological signs	Number of rats	L4		C5		Cerebellum Inflammation score	Brainstem Inflammation score	Cerebrum Inflammation score
			Inflammation score ^b	Number of apoptotic cells ^c	Inflammation score	Number of apoptotic cells			
Controls	1 (10) ^a	1	1	0	1	0	0	1	0
	2 (11.3)	3	5±1	3±1	1±0	0	0	1±0	0
	3 (13)	1	6	13	6	7	0	2	0
Re-challenged	1 (6)	1	3	14	10	81	4	3	3
	2 (6.7)	3	3±1	14±6	9±1	84±10	2±2	4±1	3±1
	3 (7)	1	3	18	9	54	2	3	2

^a Number of days after active immunization.

^b Inflammation in the meninges and parenchyma was graded on a scale of 0 (no inflammatory cells present) to 10 (severe inflammation).

^c The number of apoptotic cells in the parenchyma of the whole transverse section.

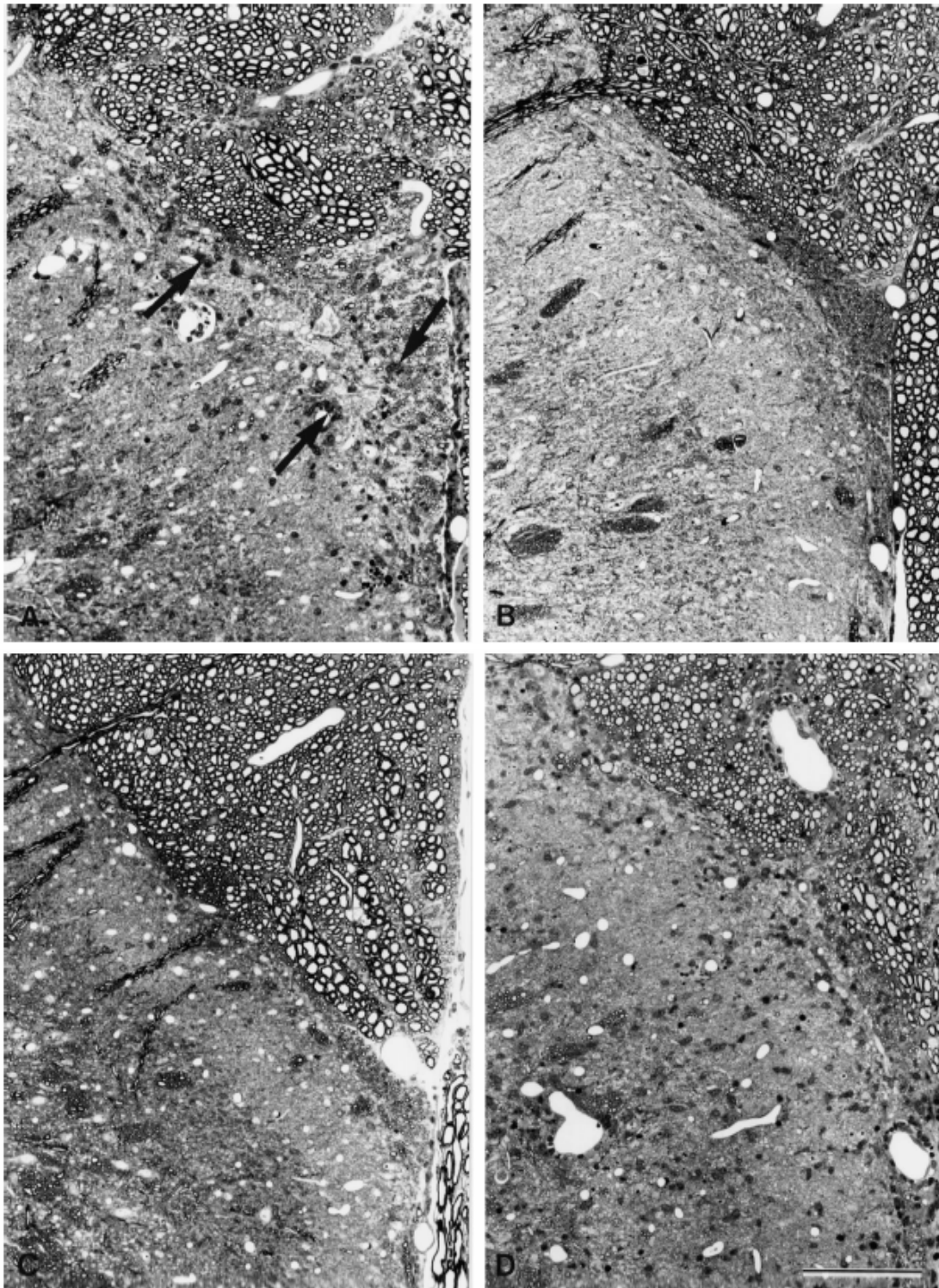


Fig. 2. Light micrographs of: (A) the lumbar spinal cord of an actively immunized naive control rat on the second day of neurological signs, showing numerous inflammatory cells (arrows) in the parenchyma; (B) the lumbar spinal cord of a rechallenged rat on the second day of neurological signs, showing minimal inflammation; (C) the cervical spinal cord of the same control rat showing no inflammation; (D) the cervical spinal cord of the same rechallenged rat showing numerous inflammatory cells throughout the parenchyma. Epoxy resin sections (0.5 μ m) stained with toluidine blue. Bar=100 μ m.

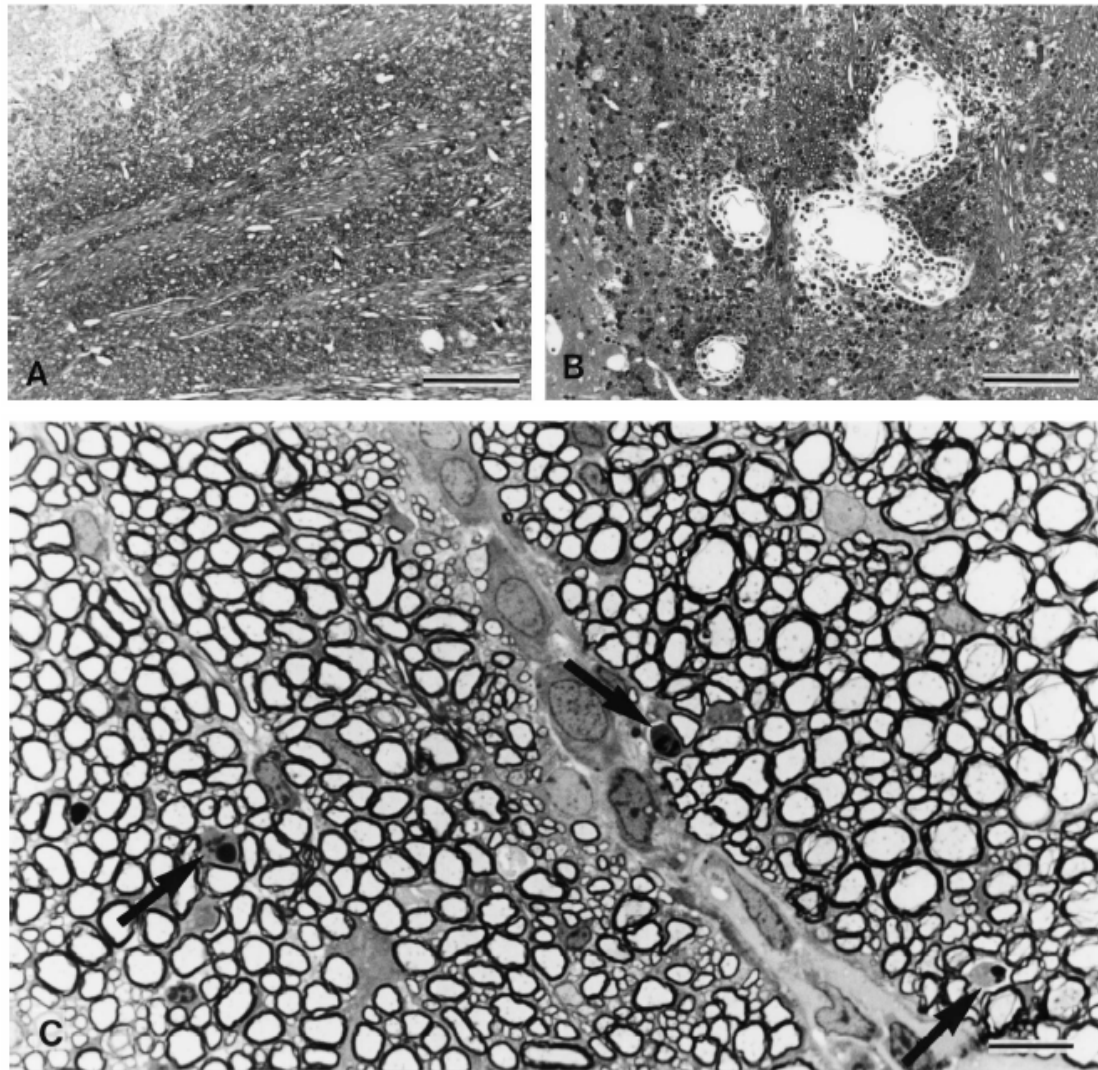


Fig. 3. (A, B) Light micrographs of the cerebellar white matter of (A) an actively immunized naive control rat on the second day of neurological signs and of (B) a rechallenged rat on the first day of neurological signs, showing no inflammation in the former and prominent inflammation in the latter. (C) Light micrograph of the lumbar spinal cord of a rechallenged rat on the second day of neurological signs showing frequent apoptotic cells (arrows) in a minimally inflamed parenchyma. (A) and (B) HistoResin sections (1 µm) stained with cresyl fast violet; (C) epoxy resin section (0.5 µm) stained with toluidine blue. Bars: (A) & (B)=100 µm; (C)=10 µm.

3.3. Flow cytometric studies of the spinal cord

The numbers of total inflammatory cells and $\text{TCR}\alpha\beta^+$ T cells isolated from the cervicothoracic cord in rechallenged rats on day 6 were higher than in control rats on days 11 ($P=0.004$ and 0.13 , respectively) and 12 ($P=0.03$ and 0.046 , respectively) (Fig. 4A–D), indicating enhanced entry of T cells. In controls, the numbers of total inflammatory cells, $\text{TCR}\alpha\beta^+$ T cells and $\text{V}\beta 8.2^+$ T cells isolated from the cervicothoracic cord were significantly lower than those isolated from the lumbosacral cord on each of the days studied ($P<0.05$), except for $\text{V}\beta 8.2^+$ T cells on day 12 ($P=0.07$) (Fig. 4A, C, E). In contrast, throughout the disease episode in rechallenged animals, the numbers of total inflammatory cells, $\text{TCR}\alpha\beta^+$ T cells and $\text{V}\beta 8.2^+$ T cells isolated from the cervicothoracic spinal cord were not significantly different from those extracted from the lumbosacral cord ($P>0.05$) (Fig. 4B, D, F). It is also clear from Fig. 4 that, whereas the numbers of total inflammatory cells and $\text{TCR}\alpha\beta^+$ T cells in the spinal cord increased early in the clinical course in controls, they began to decline on day 7, the day after the onset of neurological signs, in rechallenged animals. This decline was more dramatic in the lumbosacral cord. On day 9, only 22% of the number of total inflammatory cells present in the lumbosacral cord on day 6 remained, but in the cervicothoracic cord the corresponding percentage was 52%. In contrast to controls, there was also a rapid decline in the number of $\text{V}\beta 8.2^+$ T cells in the spinal cord, particularly

the lumbosacral cord, of rechallenged rats over the first 2 days of neurological signs (Fig. 4E, F). The number of $V\beta 8.2^+$ T cells in the lumbosacral cord was much lower in rechallenged rats than in controls.

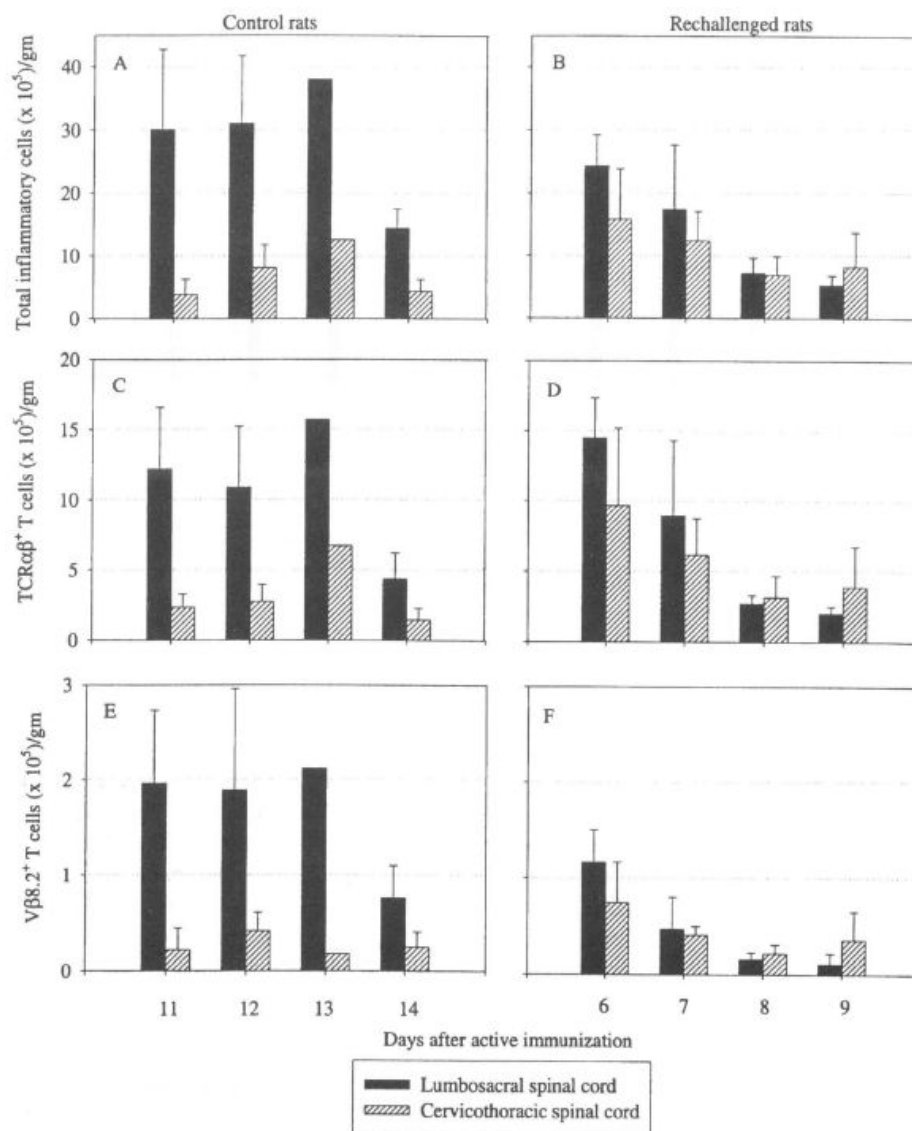


Fig. 4. The numbers of total inflammatory cells, TCRαβ⁺ T cells and Vβ8.2⁺ T cells extracted from the lumbosacral and cervicothoracic spinal cord of control rats (A, C, E), 11–14 days, and rechallenged rats (B, D, F) 6–9 days after immunization with MBP in complete Freund's adjuvant. On each of the days studied, results were obtained from 3 to 5 groups, consisting of 4–6 rats, except for day 13 for the controls when results were obtained from only one group of 6 rats.

The proportions of TCRαβ⁺ T cells in both the lumbosacral spinal cord (60.1±6.0) and cervicothoracic spinal cord (59.7±5.8) on day 6 in rechallenged rats were higher than in control rats on day 11 (41.2±4.5 and 38.8±6.3, respectively) and on all of the other days tested. However, in the lumbosacral cord of rechallenged rats this proportion dropped rapidly to be the same as in controls within 24 h after the onset of neurological signs. The proportions of Vβ8.2⁺ T cells in the CNS infiltrate were generally lower in rechallenged rats than in controls, and as a result there was a marked difference, in the proportion of TCRαβ⁺ T cells that were Vβ8.2⁺, between control and rechallenged groups (Fig. 5). Vβ8.2⁺ T cells constituted 6–9% of the TCRαβ⁺ T cell population in the spinal cord in rechallenged rats (Fig. 5B) compared with 14–17% in control rats (Fig. 5A), with the exception of the results from the cervicothoracic cord on day 13 in the latter group.

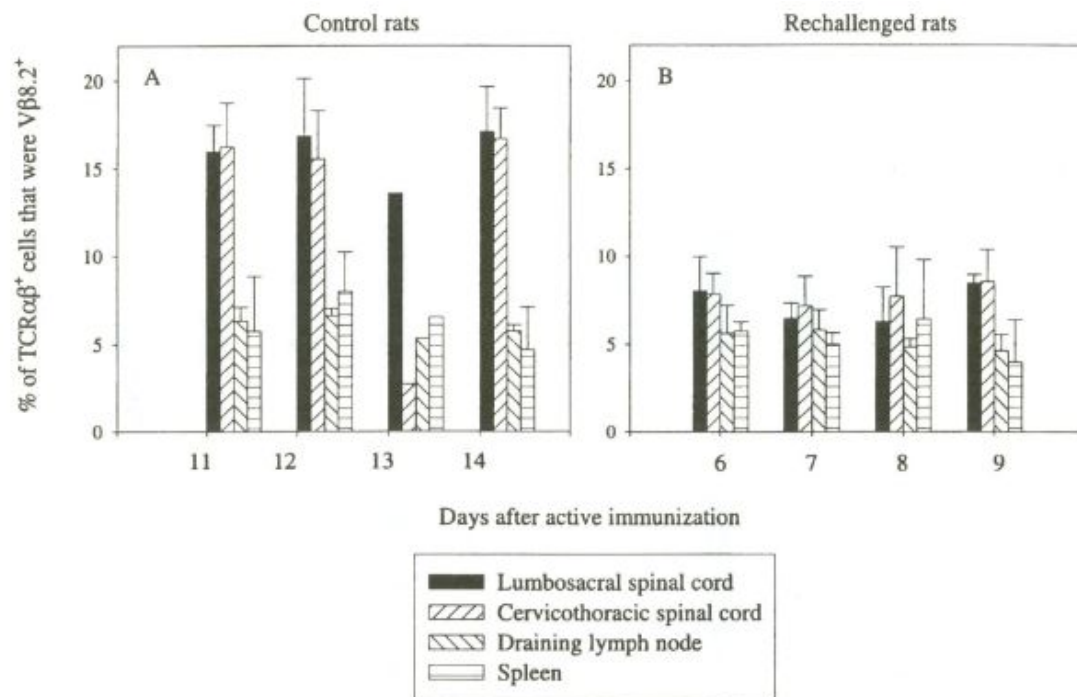


Fig. 5. Vβ8.2⁺ T cells as a proportion of the TCRαβ⁺ T cells in the lumbosacral spinal cord, cervicothoracic spinal cord, spleen and draining lymph node of control (A) and rechallenge rats (B).

The levels of apoptosis in the spinal cord infiltrate early in the clinical course were higher in rechallenge (Fig. 6B) than control rats (Fig. 6A). The highest percentage of apoptotic cells was detected on day 6 in the lumbosacral cord of rechallenge rats. We did not detect differences in the proportions of TCRαβ⁺ cells or Vβ8.2⁺ T cells that were apoptotic between control and rechallenge groups. There was also no significant difference in the enrichment of Vβ8.2⁺ T cells in the apoptotic TCRαβ⁺ population between control and rechallenge rats.

3.4. Phenotypic analysis of draining lymph node and spleen cells

The proportions of TCRαβ⁺ T cells that were Vβ8.2⁺ in the spleen and draining lymph node were similar in the control and rechallenge rats and were in the range of 4–8% (Fig. 5). Therefore, in actively induced EAE in control rats, Vβ8.2⁺ T cells are highly enriched in the inflammatory infiltrate in the spinal cord compared to the peripheral lymphoid organs, whereas in rechallenge rats there is only a slight enrichment of Vβ8.2⁺ T cells in the spinal cord infiltrate.

3.5. Limiting dilution analysis

As shown in Table 2, rechallenge rats exhibited a memory T cell response to immunization so that the frequencies of MBP-reactive T cells and MBP_{72–89}-reactive T cells in the draining lymph node of rechallenge rats 5 days after active immunization were comparable to the frequencies present in the draining lymph node of control rats on day 9. The memory T cell response in rechallenge rats was also reflected in the spleen, where the frequencies of MBP-reactive T cells and MBP_{72–89}-reactive T cells on day 5 were significantly higher than in control groups on days 5 and 9.

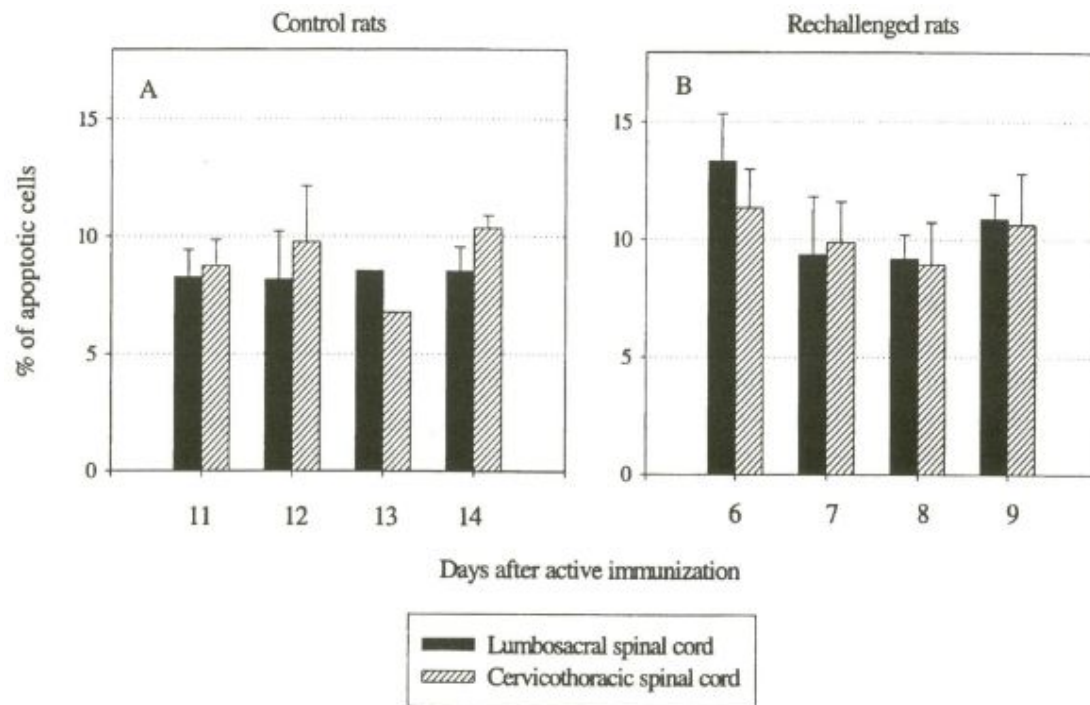


Fig. 6. The percentages of apoptotic cells in the inflammatory infiltrate isolated from the lumbosacral and cervicothoracic spinal cord of control and rechallenged rats. Lumbosacral spinal cord: rechallenge day 6 vs. controls day 11, $P=0.003$; rechallenge day 6 vs. controls day 12, $P=0.007$; rechallenge day 6 vs. controls day 14, $P=0.004$. Cervicothoracic spinal cord: rechallenge day 6 vs. controls day 11, $P=0.03$; rechallenge day 6 vs. controls day 12, $P=0.28$; rechallenge day 6 vs. controls day 14, $P=0.30$.

TABLE 2. Frequencies of MBP-, MBP₇₂₋₈₉- and MBP₈₇₋₉₉-reactive T cells in the peripheral lymphoid organs

Group of rats	Days after active immunization	Number of rats (number of experiments)	Draining popliteal lymph node			Spleen cells		
			MBP	MBP ₇₂₋₈₉	MBP ₈₇₋₉₉	MBP	MBP ₇₂₋₈₉	MBP ₈₇₋₉₉
Controls	5	13 (5) ^a	11 ^b (7-23) ^c	<7	<7	<7	<7	<7
	9	9 (3)	20 (12-35)	22 (15-35)	<7	<7	<7	<7
	11	7 (1)	46 (35-60)	46 (35-61)	<7	39 (29-52)	82 (63-108)	<7
	13	6(1)	524 (386-712)	151 (115-198)	<7	228 (172-302)	88 (67-115)	<7
Re-challenged	5	13 (5) ^a	23 (13-40)	11 (5-24)	<7	31 (20-49)	60 (45-80)	<7
	6	7 (1)	58 (44-76)	11 (7-16)	<7	80 (60-106)	33 (22-48)	<7
	8	8 (1)	101 (76-133)	54 (38-75)	<7	38 (23-61)	35 (22-56)	27 (16-46)

^a Precursor frequencies in the spleen on day 5 were determined from a total of 11 animals in four experiments.

^b Frequency per 10⁶ T cells.

^c 95% confidence limits.

In control rats the frequencies of MBP-reactive T cells and MBP₇₂₋₈₉-reactive T cells in the draining lymph node, spleen, lumbosacral spinal cord and cervicothoracic spinal cord increased between days 11 and 13 (Table 2 and Table 3). In the rechallenge rats the frequencies of MBP-reactive T cells and

MBP₇₂₋₈₉-reactive T cells increased in the draining lymph node from days 5 to 8, decreased in the lumbosacral cord from days 6 to 8 and increased in the cervicothoracic cord from days 6 to 8 (Table 2 and Table 3). This early decrease in the frequency of MBP₇₂₋₈₉-reactive T cells in the lumbosacral cord corresponds with the rapid loss of V β 8.2⁺ T cells from this region of the spinal cord (Fig. 4F) and is further evidence of rapid downregulation of the immune response in the CNS. At the peak of neurological signs in rechallenged animals, on day 8, the frequencies of MBP₇₂₋₈₉-reactive T cells in the draining lymph node and spleen were significantly lower than in control rats on day 13 (Table 2). These results suggest that, although rechallenged rats demonstrated a memory T cell response, the generation of encephalitogenic T cells in the peripheral lymphoid organs during the disease course was reduced compared with that in control animals.

TABLE 3. Frequencies of MBP-, MBP₇₂₋₈₉- and MBP₈₇₋₉₉-reactive T cells in the spinal cord

Group of rats ^a	Days after active immunization	Lumbosacral spinal cord			Cervicothoracic spinal cord		
		MBP	MBP ₇₂₋₈₉	MBP ₈₇₋₉₉	MBP	MBP ₇₂₋₈₉	MBP ₈₇₋₉₉
Controls	11	50 ^b (34–74) ^c	126 (94–170)	<7	<7	<7	<7
	13	1261 (949–1678)	1000 (755–1325)	7 (3–17)	1131 (855–1497)	670 (510–879)	11 (5–23)
Re-challenged	6	890 (675–1174)	185 (140–245)	<7	208 (157–276)	34 (20–56)	<7
	8	111 (81–152)	44 (28–70)	9 (3–23)	340 (260–445)	178 (135–236)	12 (6–24)

^a Same groups as presented in Table 2.

^b Frequency per 10⁶ T cells.

^c 95% confidence limits.

T cells reactive to MBP₈₇₋₉₉ were detectable in the lumbosacral and cervicothoracic spinal cord of control rats on day 13 and in the lumbosacral and cervicothoracic spinal cord and spleen of rechallenged rats on day 8 (Table 2 and Table 3).

3.6. Passive transfer of EAE by splenocytes from rechallenged rats

To determine whether the immune cells from rechallenged rats could induce EAE with an altered pattern of neurological signs, we transferred concanavalin-A-activated splenocytes from rechallenged rats into naive rats. In the first experiment four rats each received 75×10⁶ splenocytes that had been isolated from rechallenged rats 5 days after immunization with MBP inoculum. The recipients developed isolated tail weakness. In a second experiment we injected three rats with 150×10⁶ splenocytes isolated from rechallenged rats 6 days after immunization. These recipients developed tail paralysis, moderate hindlimb weakness and no forelimb weakness. We therefore concluded that EAE induced by immune cells from rechallenged rats follows a typical ascending course.

4. Discussion

In this study we have shown that an episode of passively transferred EAE modifies a subsequent actively induced attack of EAE through an interaction of the following mechanisms: a memory T cell response to MBP; facilitated entry of T cells into the CNS; rapid downregulation of the immune response in the CNS, with increased apoptosis of inflammatory cells; and a limited generation of encephalitogenic T cells in the peripheral lymphoid organs.

Active immunization with MBP and complete Freund's adjuvant 28 days after the passive transfer of MBP-sensitized spleen cells induced a second episode of EAE, which occurred earlier than in naive control animals, but was less severe, as previously reported (Hinrichs et al., 1981; Namikawa et al., 1986). We also observed a redistribution of the neurological signs in the rechallenged rats, with less severe tail weakness and much less severe hindlimb weakness but increased forelimb weakness, compared to controls. Our histological studies revealed an earlier onset of inflammatory CNS lesions in rechallenged rats, confirming the findings of Levine and Saltzman (1991). We also found that, early in

the clinical course in rechallenged rats, inflammation was more severe in the cervical spinal cord than in the lumbar cord, whereas the opposite was the case in controls. Furthermore, inflammation was also more severe in the cerebellum, brainstem and cerebrum in rechallenged rats than in controls. The rostral redistribution of histological lesions after the reinduction of EAE has previously been reported (Levine et al., 1967; Willenborg, 1979; Levine and Sowinski, 1980; Namikawa et al., 1986). Alterations in the distribution of CNS lesions cannot be explained by systemic mechanisms but can be explained by local factors within the CNS. We hypothesized that changes within the CNS, induced by the first episode of EAE, might explain the redistribution of neurological signs and lesions and the mild clinical course in rechallenged rats.

Limiting dilution assays of the draining popliteal lymph node and spleen revealed a memory T cell response to MBP and MBP₇₂₋₈₉ in rechallenged rats. This may contribute to the early onset of EAE. However, a memory T cell response cannot solely explain the rapid infiltration of inflammatory cells into the CNS after rechallenge, because the numbers of $\alpha\beta$ T cells and V β 8.2⁺ T cells infiltrating the cervicothoracic spinal cord were higher 6 days after rechallenge than 13 days after active immunization in naive animals, despite the fact that the T cell reactivities to MBP and MBP₇₂₋₈₉ in the draining lymph node and spleen were considerably higher in the latter. Therefore, it is likely that changes in the CNS induced by the first episode of EAE contribute to the rapid entry of inflammatory cells after rechallenge. Such changes may include disruption of the blood-brain barrier and the increased expression of adhesion molecules and chemokines.

Despite the memory T cell response and the enhanced entry of inflammatory cells into the spinal cord, the mean total clinical score at the peak of disease was lower for rechallenged rats than controls, suggesting the occurrence of downregulatory mechanisms. The rapid decline in the numbers of $\alpha\beta$ T cells and V β 8.2⁺ T cells and in the frequencies of MBP-reactive T cells and MBP₇₂₋₈₉-reactive T cells in the lumbosacral cord of the rechallenged rats indicates rapid downregulation of the immune response in this previously inflamed region of the spinal cord. This is likely to have contributed to the reduced disease in the lumbosacral cord and the reduced tail and hindlimb involvement in the rechallenged rats. The less intense downregulation in the cervicothoracic cord may have allowed the memory T cell response and increased influx of inflammatory cells to be manifested by increased cervicothoracic cord disease, which may have contributed to the increased forelimb weakness. Our observation that the passive transfer, to naive rats, of spleen cells from rechallenged rats with an altered distribution of neurological signs induced EAE with a standard pattern of neurological signs without redistribution is further evidence that this modulation of the immune response in rechallenged rats is occurring in the previously inflamed CNS and is not a feature of the encephalitogenic T cells themselves.

Our flow cytometric studies revealed that the proportion of cells undergoing apoptosis in the CNS inflammatory infiltrate was increased in the cervicothoracic spinal cord and particularly the lumbosacral cord on the day of onset of neurological signs in rechallenged rats, compared to controls. Our histological studies also indicated that the proportion of inflammatory cells undergoing apoptosis in the parenchyma of the lumbar spinal cord was higher in rechallenged rats than in control rats. This apoptosis is likely to contribute to the downregulation of the immune response in the spinal cord in rechallenged rats, as it does during the first attack of EAE (Tabi et al., 1994, 1995). Increased T cell apoptosis in the CNS in rechallenged rats could be due to increased activation-induced apoptosis as a result of increased class II major histocompatibility complex molecule expression on microglia and increased availability of MBP in the previously inflamed CNS, as we have recently suggested (White et al., 1998b). During the first attack of EAE, V β 8.2⁺ T cells, which constitute the predominant encephalitogenic MBP-reactive T cell population in the Lewis rat (Imrich et al., 1995), selectively undergo apoptosis in the CNS (Tabi et al., 1994; McCombe et al., 1996). In the present study we did not find an increase in the selective apoptosis of V β 8.2⁺ T cells in the spinal cord in rechallenged rats compared to controls. However, as there was only a low level of enrichment of V β 8.2⁺ T cells in the spinal cord in rechallenged rats compared to controls, it is likely that many of the V β 8.2⁺ T cells in the CNS of rechallenged rats were not MBP-specific and would not undergo activation-induced apoptosis in the CNS. Moreover, rapid downregulation of the TCR after reactivation in the CNS prior to apoptosis may have resulted in many apoptotic V β 8.2⁺ T cells being unable to be detected by antibody binding to V β 8.2. This is supported by our finding that T cells expressing low levels of TCR $\alpha\beta$ or V β 8.2 were much more vulnerable to apoptosis than T cells expressing high levels of TCR $\alpha\beta$ or V β 8.2 (Gordon and Pender, unpublished results).

Although there was a memory T cell response in the peripheral lymphoid organs in the rechallenged rats, the frequencies of MBP-reactive T cells and MBP₇₂₋₈₉-reactive T cells in the draining lymph node at the time of maximal neurological signs were substantially lower than in controls, indicating limited peripheral generation of encephalitogenic T cells. This might be due to a high level of interferon- γ -induced nitric oxide production (Ding et al., 1988) in the draining lymph node, as a result of the reactivation of memory T cells specific for mycobacterial antigens or MBP. Such a limited generation of encephalitogenic T cells in the periphery would fail to compensate for the apoptotic elimination of these cells in the CNS and could contribute to the lower frequency of MBP₇₂₋₈₉-reactive T cells and lower proportion of V β 8.2⁺ T cells in the CNS and to the overall reduction in disease severity in rechallenged rats.

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References

- Deibler, G.E., Martenson, R.E. and Kies, M.W., 1972. Large scale preparation of myelin basic protein from central nervous tissue of several mammalian species. *Prep. Biochem.* 2, pp. 139–165.
- Ding, A.H., Nathan, C.F. and Stuehr, D.J., 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141, pp. 2407–2412.
- Hinrichs, D.J., Roberts, C.M. and Waxman, F.J., 1981. Regulation of paralytic experimental allergic encephalomyelitis in rats: susceptibility to active and passive disease reinduction. *J. Immunol.* 126, pp. 1857–1862.
- Imrich, H., Kugler, C., Torres-Nagel, N., Dörries, R. and Hünig, T., 1995. Prevention and treatment of Lewis rat experimental allergic encephalomyelitis with a monoclonal antibody to the T cell receptor V β 8.2 segment. *Eur. J. Immunol.* 25, pp. 1960–1964.
- Kerr, J.F.R., Winterford, C.M. and Harmon, B.V., 1994. Morphological criteria for identifying apoptosis. In: Celis, J.E., Editor, 1994. *Cell Biology: A Laboratory Handbook*, Academic Press, San Diego, pp. 319–329.
- Levine, S. and Saltzman, A., 1991. Accelerated response to reinoculation in experimental allergic encephalomyelitis: Histopathologic study. *J. Neuropathol. Exp. Neurol.* 50, pp. 126–133.
- Levine, S. and Sowinski, R., 1980. Experimental allergic encephalomyelitis. Inhibition of clinical signs and paradoxical enhancement of lesions in second attacks. *Am. J. Pathol.* 101, pp. 375–385.
- Levine, S., Hoenig, E.M. and Wenk, E.J., 1967. Altered distribution of lesions after repeated passive transfers of allergic encephalomyelitis. *Proc. Soc. Exp. Biol. Med.* 126, pp. 454–458.
- Levine, S., Sowinski, R. and Steinetz, B., 1980. Effects of experimental allergic encephalomyelitis on thymus and adrenal: Relation to remission and relapse. *Proc. Soc. Exp. Biol. Med.* 165, pp. 218–224.
- Lou, Y.-H., McElveen, F., Adams, S. and Tung, K.S.K., 1995. Altered target organ. A mechanism of postrecovery resistance to murine autoimmune ophoritis. *J. Immunol.* 155, pp. 3667–3673.
- MacPhee, I.A., Antoni, F.A. and Mason, D.W., 1989. Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. *J. Exp. Med.* 169, pp. 431–445.
- McCombe, P.A., Nickson, I., Tabi, Z. and Pender, M.P., 1996. Apoptosis of V β 8.2⁺ T lymphocytes in the spinal cord during recovery from experimental autoimmune encephalomyelitis induced in Lewis rats by inoculation with myelin basic protein. *J. Neurol. Sci.* 139, pp. 1–6.
- Mostarica-Stojkovic, M., Vukmanovic, S., Ramic, Z. and Lukic, M.L., 1992. Evidence for target tissue regulation of resistance to the induction of experimental allergic encephalomyelitis in AO rats. *J. Neuroimmunol.* 41, pp. 97–104.
- Namikawa, T., Kunishita, T. and Tabira, T., 1986. Modulation of experimental allergic encephalomyelitis (EAE): Suppression of active reinduction of EAE in rats recovered from passively transferred disease. *J. Neuroimmunol.* 12, pp. 235–245.
- Nguyen, K.B. and Pender, M.P., 1989. Assessment of demyelination in glycol methacrylate sections: A new protocol for cresyl fast violet staining. *Stain Technol.* 64, pp. 163–167.

- Nguyen, K.B., McCombe, P.A. and Pender, M.P., 1994. Macrophage apoptosis in the central nervous system in experimental autoimmune encephalomyelitis. *J. Autoimmun.* 7, pp. 145–152.
- O'Brien, N.C., Charlton, B., Cowden, W.B. and Willenborg, D.O., 1999. Nitric oxide plays a critical role in the recovery of Lewis rats from experimental autoimmune encephalomyelitis and the maintenance of resistance to reinduction. *J. Immunol.* 163, pp. 6841–6847.
- Offner, H., Hashim, G. and Vandenbark, A.A., 1987. Response of rat encephalitogenic T lymphocyte lines to synthetic peptides of myelin basic protein. *J. Neurosci. Res.* 17, pp. 344–348.
- Offner, H., Hashim, G.A., Celnik, B., Galang, A., Li, X.B., Burns, F.R., Shen, N., Heber-Katz, E. and Vandenbark, A.A., 1989. T cell determinants of myelin basic protein include a unique encephalitogenic I-E-restricted epitope for Lewis rats. *J. Exp. Med.* 170, pp. 355–367.
- Panitch, H.S. and McFarlin, D.E., 1977. Experimental allergic encephalomyelitis: enhancement of cell-mediated transfer by concanavalin A. *J. Immunol.* 119, pp. 1134–1137.
- Pender, M.P., 1986. Ascending impairment of nociception in rats with experimental allergic encephalomyelitis. *J. Neurol. Sci.* 75, pp. 317–328.
- Pender, M.P., 1995. Experimental autoimmune encephalomyelitis. In: Pender, M.P. and McCombe, P.A., Editors, 1995. *Autoimmune Neurological Disease*, Cambridge University Press, Cambridge, pp. 26–88.
- Pender, M.P., Nguyen, K.B., McCombe, P.A. and Kerr, J.F.R., 1991. Apoptosis in the nervous system in experimental allergic encephalomyelitis. *J. Neurol. Sci.* 104, pp. 81–87.
- Pender, M.P., McCombe, P.A., Yoong, G. and Nguyen, K.B., 1992. Apoptosis of $\alpha\beta$ T lymphocytes in the nervous system in experimental autoimmune encephalomyelitis: its possible implications for recovery and acquired tolerance. *J. Autoimmun.* 5, pp. 401–410.
- Pender, M.P., Tabi, Z., Nguyen, K.B. and McCombe, P.A., 1995. The proximal peripheral nervous system is a major site of demyelination in experimental autoimmune encephalomyelitis induced in the Lewis rat by a myelin basic protein-specific T cell clone. *Acta Neuropathol. (Berl.)* 89, pp. 527–531.
- Schmied, M., Breitschopf, H., Gold, R., Zischler, H., Rothe, G., Wekerle, H. and Lassmann, H., 1993. Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. Evidence for programmed cell death as a mechanism to control inflammation in the brain. *Am. J. Pathol.* 143, pp. 446–452.
- Sun, D., Qin, Y., Chluba, J., Epplen, J.T. and Wekerle, H., 1988. Suppression of experimentally induced autoimmune encephalomyelitis by cytolytic T-T cell interactions. *Nature* 332, pp. 843–845.
- Swierkosz, J.E. and Swanborg, R.H., 1975. Suppressor cell control of unresponsiveness to experimental allergic encephalomyelitis. *J. Immunol.* 115, pp. 631–633.
- Tabi, Z., McCombe, P.A. and Pender, M.P., 1994. Apoptotic elimination of V β 8.2⁺ cells from the central nervous system during recovery from experimental autoimmune encephalomyelitis induced by the passive transfer of V β 8.2⁺ encephalitogenic T cells. *Eur. J. Immunol.* 24, pp. 2609–2617.
- Tabi, Z., McCombe, P.A. and Pender, M.P., 1995. Antigen-specific down-regulation of myelin basic protein-reactive T cells during spontaneous recovery from experimental autoimmune encephalomyelitis: Further evidence of apoptotic deletion of autoreactive T cells in the central nervous system. *Int. Immunol.* 7, pp. 967–973.
- Torres-Nagel, N.E., Gold, D.P. and Hünig, T., 1993. Identification of rat TCR β -V8.2, 8.5, and 10 gene products by monoclonal antibodies. *Immunogenetics* 37, pp. 305–308.
- Welch, A.M., Holda, J.H. and Swanborg, R.H., 1980. Regulation of experimental allergic encephalomyelitis. II. Appearance of suppressor cells during the remission phase of the disease. *J. Immunol.* 125, pp. 186–189.
- White, C.A., McCombe, P.A. and Pender, M.P., 1998. Microglia are more susceptible than macrophages to apoptosis in the central nervous system in experimental autoimmune encephalomyelitis through a mechanism not involving Fas (CD95). *Int. Immunol.* 10, pp. 935–941.
- White, C.A., McCombe, P.A. and Pender, M.P., 1998. The roles of Fas, Fas ligand and Bcl-2 in T cell apoptosis in the central nervous system in experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 82, pp. 47–55.
- White, C.A., Nguyen, K.B. and Pender, M.P., 2000. B cell apoptosis in the central nervous system in experimental autoimmune encephalomyelitis: roles of B cell CD95, CD95L and Bcl-2 expression. *J. Autoimmun.* 14, pp. 195–204.
- Willenborg, D.O., 1979. Experimental allergic encephalomyelitis in the Lewis rat: Studies on the mechanism of recovery from disease and acquired resistance to reinduction. *J. Immunol.* 123, pp. 1145–1150.