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Inflammatory Cells, Microglia and MHC Class II Antigen-Positive Cells in the Spinal Cord of Lewis Rats with Acute and Chronic Relapsing Experimental Autoimmune Encephalomyelitis

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Abstract

Chronic relapsing experimental autoimmune encephalomyelitis (CR-EAE) was induced in Lewis rats by inoculation with guinea pig spinal cord and adjuvants and treatment with low dose cyclosporin A (CsA). Acute EAE was induced by the same method without CsA treatment. Immunocytochemistry and flow cytometry were used to assess inflammatory cells and MHC class II (Ia) antigen expression in the central nervous system of these rats. The inflammatory infiltrate was composed mainly of $CD4^+$ T cells and macrophages, and $\alpha\beta$ T cells constituted about 65% of the $CD2^+$ T cells. After recovery from acute EAE and during the first remission of CR-EAE, the number of T cells was significantly less than in the preceding episodes. The number of T cells was higher in the second episode of CR-EAE than in the first remission. Throughout the course of CR-EAE, the majority of the $CD2^+$ T cells were $CD45RC^-$. The ratio of $IL\text{-}2R^+$ cells to $CD2^+$ cells ranged from 10.5 to 24.0%. The ratio of $CD4^+$ T cells to B cells was lower in the later episodes of CR-EAE than in the first episode. Ia antigen was expressed on infiltrating round cells at all stages of CR-EAE and on microglial cells (identified by dendritic morphology) with increasing intensity throughout the course of CR-EAE. With flow cytometry, the number of Ia^+ cells obtained from the spinal cord rose throughout the course of CR-EAE. The number of $FSC^{low}OX1^{low}$ cells, which we consider represent microglia, also increased during the course of CR-EAE.

Keywords
CD45RC; Cyclosporin A; experimental autoimmune encephalomyelitis; EAE; flow cytometry; macrophage; MHC class II; microglia; relapse; T lymphocyte

1. Introduction

In the Lewis rat, experimental autoimmune encephalomyelitis (EAE) is usually an acute monophasic illness; chronic relapsing EAE (CR-EAE) can be induced by inoculation with guinea pig spinal cord and adjuvants and treatment with low dose cyclosporin A (CsA) (Polman et al., 1988). We have previously re-ported the neuropathological and electrophysiological findings in this model of CR-EAE (Pender et al., 1990; Stanley and Pender, 1991). The means by which low dose CsA causes CR-EAE is not known. Polman et al. (1988) suggested that suppressor mechanisms are inactivated by CsA, or that relapses are a rebound effect after cessation of the drug. In our study of chronic relapsing experimental autoimmune neuritis induced by inoculation with intradural root myelin and adjuvants and treatment with low dose CsA, we also suggested that low dose CsA treatment interferes with the regulatory mechanisms which prevent relapses (Mc-Combe et al., 1990). One aim of the present study was to compare the immunopathology of acute EAE and CR-EAE, to search for effects of low dose CsA which might lead to relapses.

A second aim was to study the inflammatory infiltrate in the CNS during the course of CR-EAE, to determine whether it declined in clinical remission and intensified during relapse and whether the relative proportions of cells of different types varied through-out the course of CR-EAE. In the guinea pig (Traugott et al., 1982; Butter et al., 1989) and mouse (Traugott et al., 1986; Baker et al., 1990) models of CR-EAE, immunocytochemical studies have

demonstrated T cells and macrophages in the central nervous system (CNS). In CR-EAE induced in the Lewis rat by treatment with low dose CsA, Polman et al. (1988) found T cells and macrophages in the CNS 50 days after inoculation. In this model we have shown that each relapse is associated with inflammation and demyelination in the CNS (Pender et al., 1990). We have previously used immunocytochemistry and flow cytometry to study acute EAE induced by inoculation with myelin basic protein (MBP) (MBP-EAE) (McCombe et al., 1992). Such methods have not been used in CR-EAE, so in the present study we used a combination of immunocytochemistry and flow cytometry to assess inflammatory cells and MHC class II (Ia) antigen expression in the CNS throughout the course of CR-EAE.

2. Materials and methods

2.1. Animals

Lewis rats (JC strain) were obtained from the Central Animal Breeding House of The University of Queensland.

2.2. Preparation of inoculum and induction of EAE

The inoculum consisted of a mixture of 1 g guinea pig spinal cord in 1 ml of 0.9% saline emulsified with 1 ml complete Freund's adjuvant (Difco) containing an additional 10 mg *Mycobacterium tuberculosis* H37RA (Difco). As described previously (Pender et al., 1990), CR-EAE was induced by inoculation of Lewis rats with 0.05 ml inoculum into the medial footpad of the right hindfoot. Commencing on the day of inoculation, rats were given subcutaneous injections of cyclosporin A (Sandoz) 4 mg/kg on 3 days per week until day 21 after inoculation. Rats with acute EAE were inoculated as above but were not given CsA.

2.3. Clinical assessment

Tail, hindlimb and forelimb weakness were graded on a scale of 0 (no weakness) to 4 (complete paralysis) as previously described (Pender, 1986). The total clinical score was obtained by adding the scores for the tail, hindlimbs and forelimbs.

2.4. Monoclonal antibodies

The following monoclonal antibodies were obtained from Serotec and by kind donation from Dr J. Sedgwick: R73 ($\alpha\beta$ T cell receptor) (Hünig et al., 1989), OX34 (CD2 on T cells and natural killer (NK) cells) (Williams et al., 1987), OX39 (IL-2R) (Paterson et al., 1987), OX22 (CD45RC, the high molecular mass form of the leukocyte common antigen on B cells, macrophages and some T cells) (Spickett et al., 1983; Woollett et al., 1985), OX8 (CD8 on MHC class I restricted T cells and some NK cells) (Brideau et al., 1980), W3/25 (CD4) (White et al., 1978), OX33 (B cells) (Woollett et al., 1985), OX12 (kappa light chains) (Hunt and Fowler, 1981; Barclay, 1981a), OX6 (MHC class II) (McMaster and Williams, 1979; Barclay, 1981b); OX42 (CD11b) (Robinson et al., 1986) and OX1 (leukocyte common antigen on all cells of haemopoietic origin) (Sunderland et al., 1979).

2.5. Immunostaining

Rats were perfused with cold, isotonic saline. The spinal cord was removed and immediately frozen. Frozen sections (5 μm) from the lumbar and thoracic spinal cord and brainstem were collected on poly-L-lysine coated slides, air-dried, fixed in chloroform/acetone (1 : 1) and stored at –20°C until required. Endogenous peroxidase activity was blocked by 1% (w/v) phenylhydrazine. Sections were washed in phosphate-buffered saline (PBS) and incubated with normal rabbit serum. Sections

were washed in PBS, incubated with unconjugated primary mouse monoclonal antibody (ascites fluid) diluted 1:100 in PBS containing 20% foetal calf serum (FCS), washed, and exposed to peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts). Sections were incubated with diaminobenzidine for 10-15 min. Sections without primary antibody were used as controls. Cells were regarded as positively stained if the cell outline was dark brown.

2.6. Isolation of cells from the spinal cord

Rats were perfused with cold, sterile, isotonic saline. Spinal cords were removed by insufflation, weighed, rinsed in Hank's balanced salt solution (HBSS) and passed through a 200-mesh stainless steel sieve and suspended in HBSS. Cells were isolated in a Percoll gradient using a modification of the method of Cohen et al. (1987). A two-step discontinuous Percoll (Pharmacia) gradient was prepared by adding 10 ml isotonic Percoll to 24 ml spinal cord suspension and underlaying this 1.04 g/ml layer with 1.11 g/ml Percoll solution. After centrifugation at 1500 × *g* for 30 min at 4°C, cells were collected from the 1.04-1.11 interface.

2.7. Immunofluorescent labelling for flow cytometry

100-μ1 aliquots of cells suspended in PBS, 10% normal rabbit serum were incubated with 100 μl monoclonal mouse anti-rat leukocyte primary antibody (Serotec ascites antibodies were diluted 1:100 and hybridoma supernatant antibodies obtained from Dr. J. Sedgwick were diluted 1: 30) for 30 min at 4°C or on ice. Cells were washed in 1 ml cold PBS, 1% FCS, 0.1% sodium azide and centrifuged at 600 × *g* for 5 min at 4°C. The wash solution was decanted and any excess blotted. The secondary antibody was a rat IgG-absorbed affinity purified $F(ab')_2$ rabbit anti-mouse IgG phycoerythrin (PE) conjugate (Serotec). 50 μl secondary antibody diluted 1: 40 in PBS was added to the cells for 30 min at 4°C or on ice in the dark. Cells were washed as above. For two-colour procedures 100 μl fluorescein-conjugated monoclonal antibody (diluted 1:100) was incubated with cells for 30 min at 4°C or on ice in the dark. Cells were washed as above and resuspended in 0.25 ml PBS, 1% FCS, 0.1% sodium azide and stored on ice in the dark prior to flow cytometric analysis. Control cells were incubated with secondary antibody only.

2.8. Flow cytometry

Single cell immunofluorescence analysis was performed using a FACScan flow cytometer and LYSYS II software (Becton Dickinson). For two-colour procedures, compensation settings corrected for the overlap of the emission spectra of fluorescein isothiocyanate (FITC) and PE. Except for the studies with normal rat spinal cord, where few cells were obtained, data were collected for 10,000 events. Debris and dead cells were excluded by gating. The numbers of positively labelled cell populations were calculated by subtracting the equivalent control values. The number of positively labelled cells/g spinal cord was calculated by multiplying the percentage of labelled cells by the yield of cells/g of spinal cord. To confirm that the cell population was composed of live cells, we used staining with fluorescein diacetate succinymidyl ester (CFSE) (Molecular Probes) with cells being incubated in 5 μM CFSE in PBS for 15 min at 37°C at a density of approximately 10^7 cells/ml and washed twice as previously described before analysis. Dead cells were assessed by staining with propidium iodide (PI). Cells were resuspended in 5μg/ml PI in PBS and placed on ice just prior to analysis.

TABLE 1
Details of rats used in immunocytochemistry and flow cytometry

Disease	Number of rats	Day after inoculation at time of study	Mean ± SD disability score when studied	Mean ± SD yield of cells from spinal cord (cells × 10^6g
Rats studied by immunocytochemistry				
No CsA treatment				
Acute EAE	5	12-14	6.2 ± 1.3	
Recovery from acute EAE	1	20	1.0	
CsA treated				
1st episode CR-EAE	5	12-14	7.4 ± 2.3	
1st remission CR-EAE	5	18-20	0.4 ± 0.5	
2nd episode CR-EAE	5	21-25	5.8 ± 1.6	
Chronic persistent EAE	3	29-53	5.5 [a]	
Rats studied by flow cytometry				
Normal rats	5	–	–	0.17 ± 0.07
No CsA treatment				
Acute EAE	5	10-13	6.6 ± 0.8	5.0 ± 1.9
Recovery from acute EAE	4	16-21	0	4.0 ± 2.0
CsA treated				
1st episode CR-EAE	5	12-14	6.7 ± 1.1	6.5 ± 1.0
1st remission off CR-EAE	5	18-21	0.2 ± 0.4	3.0 ± 1.0
2nd episode CR-EAE	5	21-23	7.6 ± 1.6	9.1 ± 3.6
3rd episode CR-EAE	5	26-34	7.2 ± 2.0	5.6 ± 1.4

[a] This score is the mean of two rats. the third rat was moribund and the neurological assessment was considered to be unreliable.

2.9. Statistical methods

To compare the numbers of cells obtained from rats with acute EAE and rats in the first episode of CR-EAE we used Student's *t*-test. To make comparisons among the different stages of CR-EAE, we used analysis of variance (ANOVA), and performed post-analysis of variance calculations to obtain a Bonferroni *P* value, which is corrected for multiple comparisons, and which we have denoted as *P**. Significance was taken to be $P < 0.05$. Non-significant results are denoted as NS.

3. Results

3.1. Clinical observations

Inoculated rats that did not receive CsA usually had a single episode of acute EAE from which they recovered completely. As in our previous study (Pender et al., 1990), rats treated with low dose CsA developed CR-EAE, with most rats having at least three episodes of neurological signs separated by periods of remission, and a minority having persistent neurological signs of EAE (chronic persistent EAE) (Fig. 1). In general, the rats treated with low dose CsA had no further neurological episodes from about 40 days after inoculation. The clinical details of the rats in the present study are shown in Table 1.

3.2. Immunocytochemical staining

Normal rats

Occasional Ia^+ cells and occasional $TCR\alpha\beta^+$ cells were seen in the meninges and around blood vessels of the spinal cord. In the parenchyma of the spinal cord, cells with dendritic

morphology were weakly $CD11b^+$ and weakly $CD4^+$.

Acute EAE (no CsA)

Acute episode. The inflammatory infiltrate in the spinal cord and in the brainstem contained $CD2^+$ cells (Fig. 2A) and $CD11b^+$ round cells and was most prominent in the meningeal, subpial and perivascular regions. Many cells were $CD4^+$. Few CDB^+ cells, few IL-$2R^+$ cells and few B cells were present. Some cells in the parenchymal inflammatory infiltrate were strongly $CD45RC^+$. Many round cells and cells with dendritic morphology were Ia^+.

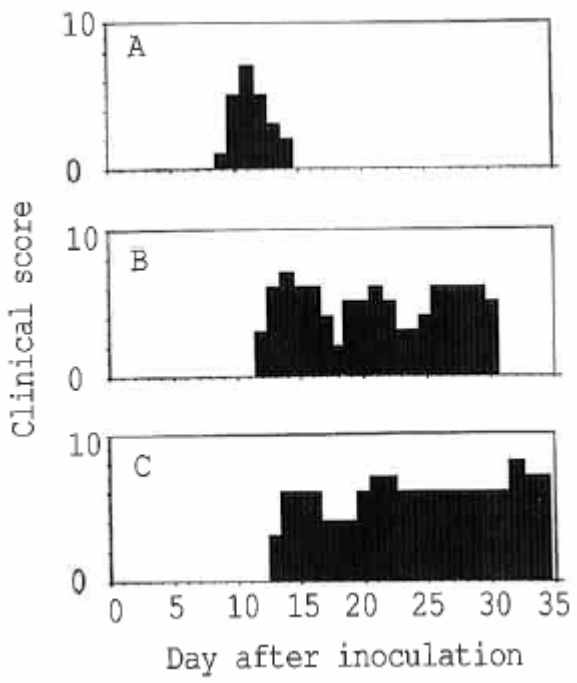

Fig. 1. Typical clinical courses of (A) a rat with acute EAE (B) a low dose cyclosporin A-treated rat with a chronic relapsing course of EAE and (C) a low dose cyclosporin A-treated rat with chronic persistent EAE. The clinical score was obtained by adding the scores for tail, hindlimb and forelimb weakness. The experiments in (B) and (C) were terminated by perfusion.

Recovery from acute EAE. The number of infiltrating cells was considerably less than in the acute episode. There were occasional $CD2^+$ cells in a subpial distribution. A few round cells in the white matter and many cells of dendritic morphology throughout the grey and white matter were $CD11b^+$. Some $CD45RC^+$ cells were present in the subpial regions, but few $CD45RC^+$ cells were present in the parenchyma of the spinal cord. Ia expression was detected on cells of dendritic morphology throughout the spinal cord and on round cells in the white matter.

CR-EAE (CsA-treated)

First episode. There was a prominent subpial infiltrate with $CD2^+$ cells and $CD11b^+$ round cells (Fig. 2A). $CD11b^+$ cells with dendritic morphology were also prominent. Many $CD4^+$ round cells were present in the meninges and white matter. $CD4^+$ cells with dendritic morphology were also seen. Few B cells or CDB^+ cells were seen. $CD45RC^+$ cells were present in the meninges, perivascular regions and in the parenchyma of the spinal cord. Many infiltrating round cells and also of cells with dendritic morphology were Ia^+. Occasional IL-$2R^+$ cells were seen.

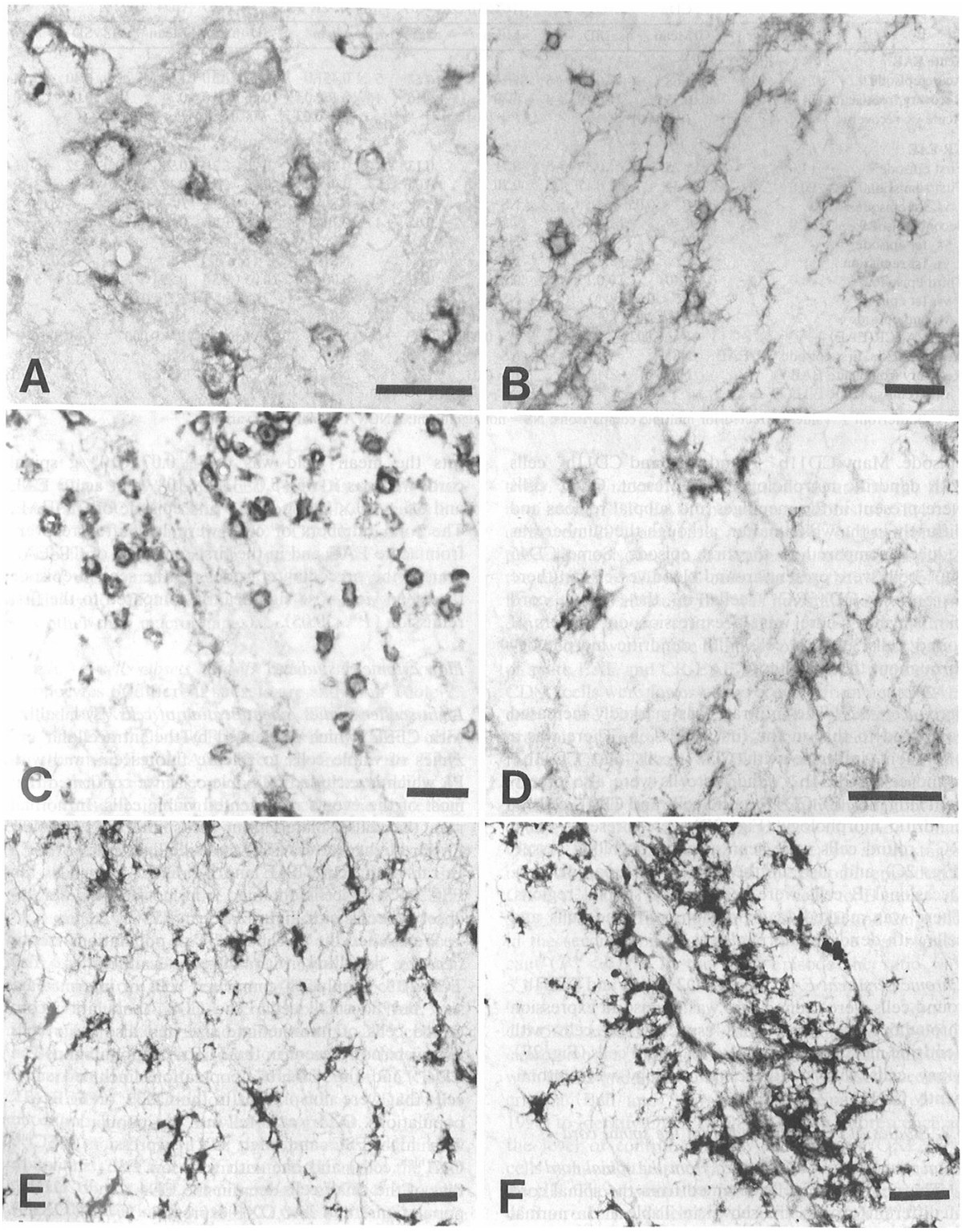

Fig. 2. Immunoperoxidase staining of frozen sections from the spinal cord of rats with CR-EAE. **(A)** $CD2^+$ round cells in the white matter of a rat in the first episode of CR-EAE, 14 days after inoculation. Bar represents 25 μm. **(B)** $CD4^+$ cells in the spinal cord of a rat in the second episode of CR-EAE, 22 days after inoculation. Bar represents 25 μm. (C) $OX22^+$ cells around a blood vessel and in adjacent white matter of a rat in the second episode of CR-EAE, 30 days after inoculation. Bar represents 25 μm. (**D**) Ia expression in the spinal cord of a rat with chronic persistent EAE, 29 days after inoculation. Bar represents 25 μm. **(E)** $CD11b^+$ cells in the spinal cord of a rat in the second episode of CR-EAE, 21 days after inoculation. Bar represents 25 μm. (**F**) $CD11b^+$ cells around a blood vessel of a rat in the second episode of CR-EAE, 22 days after inoculation. Bar represents 25 μm.

First remission. The number of infiltrating cells was considerably reduced compared to that in the first episode. Many $CD11b^+$ round cells and $CD11b^+$ cells with dendritic morphology were present. $CD2^+$ cells were present in the meninges and subpial regions and diffusely in the white matter, although the number was reduced compared to the first episode. Some CD45 RC^+ cells were present around blood vessels but there were few $CD45RC^+$ cells in the spinal cord parenchyma. There was Ia expression on clusters of round cells and on cells with dendritic morphology throughout the spinal cord.

Second episode. The infiltrate was markedly increased compared to that in the first remission. There was a prominent infiltrate with $CD2^+$ cells and $CD11b^+$ round cells. $CD1lb^+$ dendritic cells were also prominent (Fig. 2D, E). $CD4^+$ round cells and $CD4^+$ cells of dendritic morphology (Fig. 2B) were present. CD45 RC^+ round cells were seen around the blood vessels (Fig. 2C) and also in the spinal cord parenchyma. Occasional B cells were seen in the subpial regions. There was marked Ia expression by round cells and cells with dendritic morphology.

Chronic persistent EAE. Some $CD2^+$ cells and $CD11b^+$ round cells were seen. There was intense Ia expression throughout the spinal cord especially on cells with dendritic morphology, but also on round cells (Fig. 2F). Many cells with dendritic morphology were prominently $CD11b^+$.

3.3. Studies of cells extracted from the spinal cord

Total number of cells extracted from the spinal cord

The numbers of cells obtained from the spinal cord of different groups are shown in Table 1. In normal rats the mean yield was $0.17 \pm 0.07 \times 10^6$/g spinal cord, whereas it was $5.0 \pm 1.9 \times 10^6$/g in acute EAE and $6.5 \pm 1.0 \times 10^6$/g in the first episode of CR-EAE. The total numbers of cells were less after recovery from acute EAE and in the first remission of CR-EAE than in the preceding episodes. In the second episode the mean yield rose significantly compared to the first remission ($P^* < 0.05$).

Flow cytometry studies

Light scatter studies /identification of cells. By labelling with CFSE, which is cleaved by the intracellular enzymes of viable cells to release fluorescein, and with PI, which is excluded by viable cells, we confirmed that most of the events represented viable cells. In normal rats, the cells isolated from the spinal cord had low forward light scatter (FSC) and side light scatter (SSC). In rats with acute EAE and CR-EAE, as well as the $FSC^{low}SSC^{low}$ cells obtained from normal rats we also observed cells with higher FSC and SSC.

We studied the FSC and SSC of populations identified by labelling with different antibodies. The TCR$\alpha\beta^+$ population comprised cells of intermediate size, but no small cells. The $CD2^+$ population contained cells of intermediate size and also large cells that were not present in the TCR$\alpha\beta^+$ population. The $CD4^+$ and the $CD11b^+$ populations included small cells that were not present in the $CD2^+$ or TCR$\alpha\beta^+$ populations. $OX1^+$ cells fell into two populations, one with high FSC and high OX1 expression (FSC^{high} $OX1^{high}$ cells) and one with low FSC (similar to that of the small cells seen in the $CD4^+$ and $CD11b^+$ populations) and low OX1 expression ($FSC^{low}OX1^{low}$ cells) (Fig. 3). We concluded (see Discussion) that these $FSC^{low}OX1^{low}$ cells were microglia, as were the $FSC^{low}CD4^+$ cells and the $FSC^{low}CD11b^+$ cells. Double-labelling confirmed the presence of $CD11b^+$ $OX1^{high}$ cells (macrophages), $CD11b^+OX1^{low}$ cells (microglia) and $CD11b^-OX1^{high}$ cells (haematogenous cells other than macrophages).

Table 2
Numbers of cells × 10^6/g of spinal cord

	$CD2^+$			$TCR\alpha\beta^+$			$IL\text{-}2R^+$			$CD4^+CD2^+$		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Acute EAE												
Acute episode	1.68	0.8	5	1.14	0.52	5	0.45	0.21	5	1.04	0.40	5
Recovery from acute EAE	0.55	0.19	4	0.40	0.16	4	0.15	0.06	4	0.31	0.09	4
Acute vs. recovery	$P < 0.001$			$P = 0.04$			$P = 0.03$			$P < 0.01$		
CR-EAE												
First episode	1.25	0.3	5	0.71	0.13	4	0.22	0.20	3	0.91	0.22	5
First remission	0.34	0.17	5	0.20	0.06	5	0.08	0.05	5	0.17	0.08	3
vs. 1st episode	$P^* < 0.05$			NS			NS			$P^* < 0.05$		
Second episode	1.49	0.68	3	0.83	0.55	4	0.10	0.05	5	0.62	0.31	5
vs. 1st episode	NS			NS			NS			NS		
vs. 1st remission	$P^* < 0.05$			NS			NS			NS		
Third episode	0.40	0.17	5	0.25	0.09	5	0.08	0.05	5	0.29	0.12	5
vs. 1st episode	$P^* < 0.05$			NS			NS			$P^* < 0.05$		
vs. 2nd episode	$P^* < 0.05$			NS			NS			NS		
ANOVA (CR-EAE)	$P = 0.002$			$P = 0.006$			NS			$P = 0.0007$		
Acute EAE vs. first episode CR-EAE	NS			NS			NS			NS		
Recovery from acute EAE vs. first remission CR-EAE	NS			$P = 0.03$			NS			NS		

P^* = Bonferroni P value, corrected for multiple comparisons; NS = not significant; ANOVA = analysis of variance.

T cells, T cell subsets and B cells. The numbers of lymphocytes of different subsets are shown in Table 2. The majority of lymphocytes were T cells. The numbers of $CD2^+$ cells, $TCR\alpha\beta^+$ cells and $IL\text{-}2R^+$ cells are shown in Fig. 4. After recovery from acute EAE and in the first remission of CR-EAE the numbers of $CD2^+$ cells fell significantly compared to the preceding episodes ($P < 0.05$). The numbers of $TCR\alpha\beta^+$ and $IL\text{-}2R^+$ cells were less after recovery from acute EAE and in the first remission of CR-EAE than in the preceding episodes. In the second episode of CR-EAE, the numbers of $CD2^+$ cells and $TCR\alpha\beta^+$ cells were greater than in the first episode but these differences were not statistically significant. In the third episode, the numbers of $CD2^+$ cells and $TCR\alpha\beta^+$ cells were less than in the first and second episodes. There was no significant change in the numbers of $IL\text{-}2R^+$ cells in the second and third episodes compared to that in the first episode. The ratios of the numbers of $TCR\alpha\beta^+$ cells to $CD2^+$ cells and of $IL\text{-}2R^+$ cells to $CD2^+$ cells did differ significantly between the groups.

$CD4^+$ T cells were the predominant subset in all groups of rats (Table 2). In acute EAE and in the first episode of CR-EAE, the numbers of $CD4^+$ T cells were 1.04 ± 0.40 and 0.91 ± 0.22 cells × 10^6/g, respectively. After recovery from acute EAE and in the first remission of CR-EAE, the mean numbers of $CD4^+$ T cells were 29% and 18%, respectively, of those in the preceding episodes. In the second episode of CR-EAE the number of $CD4^+$ T cells was less than that in the first episode, although this was not statistically significant, while in the third episode it declined further. $CD8^+$ cells were in the minority of T cells at all stages of acute EAE and CR-EAE (Table 2). The numbers of $CD8^+$ cells were lower after recovery from acute EAE and in the first remission of CR-EAE than in the previous episodes (NS; $P^* < 0.05$, respectively). The ratio of $CD4^+$ T cells to $CD8^+$ cells was 9.1 + 2.9 in acute EAE and 9.7 ± 4.1 in the first episode of CR-EAE and did not change significantly throughout the course of CR-EAE. The numbers of $OX33^+$ and $OX12^+$ cells (B cells) (Table 2) were low at all stages of EAE but were highest in the second episode of CR-EAE. The ratio of $CD4^+$ T cells to B cells was 9.7 ± 3.9 in the first episode of CR-EAE and 2.6 ± 0.6 in the second episode and this difference was significant ($P^* < 0.05$). In the third episode the ratio was 5.3 ± 4.6.

Table 2 (cont'd)

$CD8^+$			$CD45RC^+CD2^+$			$CD45RC^+TCR\alpha\beta^+$			$OX33^+$			$OX12^+$		
Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
0.12	0.06	5	0.26	0.12	5	0.11	0.04	5	0.14	0.08	5	0.13	0.09	5
0.06	0.04	4	0.04	0.02	4	0.02	0.01	4	0.11	0.06	4	0.10	0.06	4
NS			$P = 0.009$			$P = 0.003$			NS			NS		
0.10	0.02	5	0.22	0.07	5	0.11	0.04	4	0.10	0.03	5	0.14		1
0.03	0.02	5	0.02	0.01	3	0.01	0	3	0.11	0.09	5	0.09	0.06	5
$P^* < 0.05$			$P^* < 0.05$			NS			NS					
0.10	0.05	4	0.19	0.07	3	0.07	0.05	5	0.24	0.13	5	0.23	0.13	4
NS			NS			NS			NS					
$P^* < 0.05$			NS			NS			NS					
0.04	0.02	5	0.08	0.04	5	0.06	0.02	4	0.07	0.03	4	0.06	0.03	5
NS			$P^* < 0.05$			NS			NS					
NS			NS			NS			NS					
$P = 0.0018$			$P = 0.0009$			$P = 0.027$			$P = 0.032$					
NS			NS			NS			NS					
NS			NS			NS			NS			NS		

CD45RC expression on T cells. To examine CD45RC expression on T lymphocytes we used dual labelling with OX22 and either OX34 or R73. We used accepted criteria (Bell and Sparshott, 1990; Sparshott et al., 1991) to identify $OX22^-$ cells as having fluorescence at the level of control antibody binding and $OX22^{bright}$ cells as having the same level of OX22 fluorescence as B cells. There were also cells with intermediate levels of fluorescence which are described as $OX22^{dim}$ cells (Fig. 3). We included both $OX22^{bright}$ and $OX22^{dim}$ cells in the $OX22^+$ cells. Double labelling with OX33 and OX22 was employed to establish the level of OX22 fluorescence by B cells.

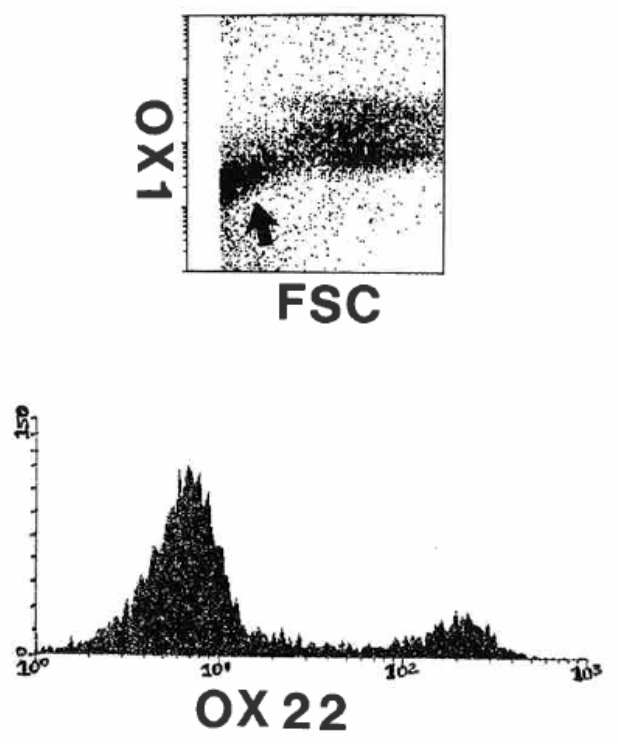

Fig. 3. Upper panel: Flow cytometry analysis of OX1 expression and -forward light scatter (FSC) of cells extracted from the spinal cord of a rat with CR-EAE. Two populations are shown. One population

(arrow) is $FSC^{low}OX1^{low}$ and we consider this population represents microglia (see Discussion). The other population is $FSC^{high}OX1^{high}$ and includes macrophages and lymphocytes. As there is some over-lap of OX1 expression between the two populations, we used FSC as well as OX1 expression to help define the two populations. Lower panel: OX22 (CD45RC) fluorescence of lymphocytes obtained from the spinal cord of a rat with acute EAE. There is a peak of low fluorescence ($0X22^{-}$cells) and a peak of high fluorescence ($OX22^{bright}$ cells). There are also some cells at intermediate levels of fluorescence which are described as $OX22^{dim}$ (Bell and Sparshott, 1990; Sparshott et al., 1991). We included both $OX22^{dim}$ and $OX22^{bright}$ cells in the $OX22^{+}$ group.

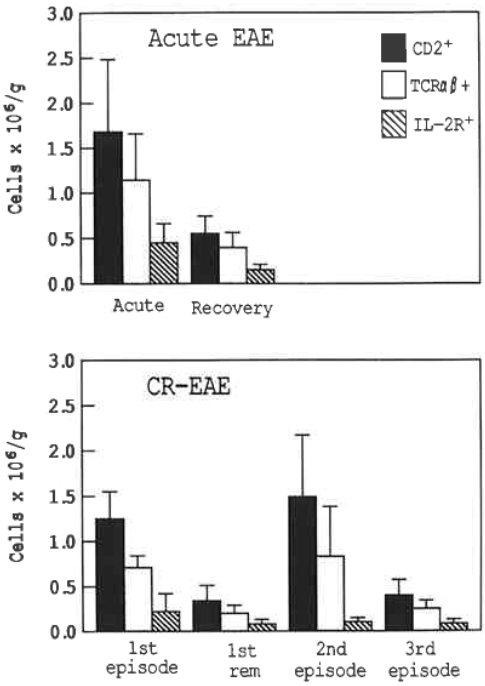

Fig. 4. Numbers of T cells of different types obtained from the spinal cord in acute EAE (upper panel) and at different stages of CR-EAE (lower panel). The number of $TCR\alpha\beta^{+}$ cells was less than the number of $CD2^{+}$ cells at all stages of disease. The number of T cells of all types declined after recovery from acute EAE and during the first remission of CR-EAE. Many T cells were obtained from the spinal cord in the second episode of CR-EAE. (Rem = remission).

Table 3 shows the frequency of $CD45RC^{+}CD2^{+}$ cells as a percentage of $CD2^{+}$ cells and the frequency of $CD45RC^{+}TCR\alpha\beta^{+}$ cells as a percentage of $TCR\alpha\beta^{+}$ cells at different stages of disease. At all stages of acute EAE and CR-EAE, the majority of T cells were $CD45RC^{-}$. The mean proportion of $CD2^{+}$ T cells that were $CD45RC^{+}$ fell after recovery from acute EAE ($P = 0.005$), as did the proportion of $TCR\alpha\beta^{+}$ cells that were $CD45RC^{+}$ ($P = 0.04$). The mean numbers of $CD2^{+}$ and $TCR\alpha\beta^{+}$ cells that were $CD45RC^{+}$ fell in the first remission of CR-EAE compared to the first episode, but not significantly.

TABLE 3
CD45RC expression on T cells

	$CD45RC^+CD2^+/CD2^+$ (%)		$CD45RC^+TCR\alpha\beta^+/TCR\alpha\beta^+$ (%)	
	Mean ± SD	*n*	Mean ± SD	*n*
Acute EAE				
Acute episode	16.2 ± 3.6	5	10.2 ± 4.0	5
After recovery from acute EAE	8.3 ± 1.7	4	4.9 ± 1.0	4
Acute vs. recovery	$P = 0.005$		$P = 0.04$	
CR-EAE				
First episode	15.1 ± 2.3	5	11.7 ± 3.0	4
First remission	11.3 ± 1.3	3	6.9 ± 1.1	3
Second episode	14.7 ± 2.4	3	8.9 ± 2.1	5
Third episode	20.7 ± 4.3	5	17.0 ± 3.8	4
	$P^* < 0.05$ vs. 1st remission		$P^* < 0.05$ vs. 1st remission $P^* < 0.05$ vs. 2nd episode	
ANOVA (all CR-EAE)	$P = < 0.006$		$P = 0.04$	
Acute EAE vs. first episode CR-EAE	NS		NS	

Macrophages and microglia. To study macrophages and microglia we used labelling with OX42 and OX1 (Table 4). We measured the numbers of $CD11b^+$ cells (macrophages and microglia), FSC^{low}O X 1 ^{low} cells (microglia), and $CD11b^+$ $OX1^{high}$ cells (macrophages). The total number of $CD11b^+$ cells was the same in acute EAE and the first episode of CR-EAE and did not fall after recovery from acute EAE or in the first remission of CR-EAE. In the second episode of CR-EAE the mean number of total $CD11b^+$ cells was greater than in the first episode ($P^* < 0.05$) and in the first remission ($P^* < 0.01$). In the third episode the number of $CD11b^+$ cells was also higher than in the first episode of CR-EAE but this was not statistically significant.

The number of $CD11b^+OX1^{high}$ cells (macrophages) did not differ significantly between acute EAE and the first episode of CR-EAE and did not fall significantly after recovery from acute EAE or in the first remission of CR-EAE. The number of $CD11b^+$ $OX1^{high}$ cells was higher in the second episode of CR-EAE than in the first remission ($P^* < 0.05$) and in the third episode it was less than in the second episode ($P^* < 0.05$). The percentage of $CD11b^+$ cells that were $OX1^{high}$ cells fell significantly after recovery from acute EAE ($P = 0.01$). In CR-EAE, the percentage of $CD11b^+$ cells that were $OX1^{high}$ declined progressively throughout the course of disease from 51.5% ± 27.6 in the first episode to 11.3% ± 2.6 in the third episode. This indicates that during the course of CR-EAE an increasing proportion of $CD11b^+$ cells were $OX1^{low}$ (microglia). This was further analysed by studies of FSC^{low} OX1 ^{low} cells (Table 4 and Fig. 5). The number of FSC^{low} $OX1^{low}$ cells was similar in acute EAE and in the first episode of CR-EAE and did not change significantly after recovery from acute EAE or in the first remission of CREAE. In the second episode of CR-EAE the number of FSC^{low} $OX1^{low}$ cells was significantly higher than in the first remission and the first episode of CR-EAE ($P^* < 0.05$ in each case) and in the third episode it remained high.

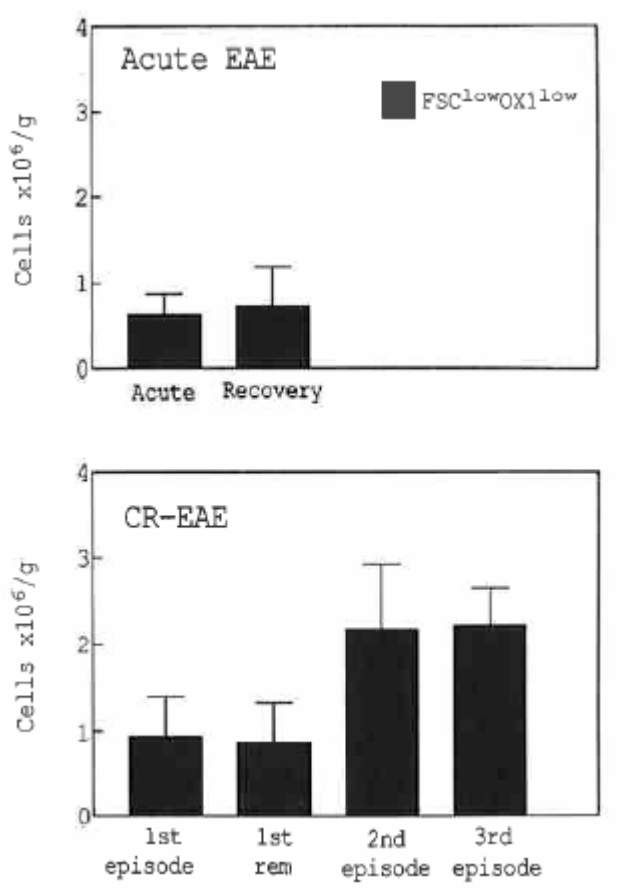

Fig. 5. Numbers of FSC ^{low} OX1 ^{low} cells (microglia) obtained from the spinal cord of rats with acute EAE and CR-EAE. The number of these cells increases throughout the course of CR-EAE. (Rem, remission).

Ia$^+$ (OX6$^+$) cells. Table 4 shows the number of Ia$^+$ cells at different stages of disease. The number of Ia$^+$ cells was the same in acute EAE and in the first episode of CR-EAE and did not change significantly after recovery from acute EAE or in the first remission of CR-EAE. It was higher in the second episode of CR-EAE than in the first episode ($P^* < 0.01$) and remained higher in the third episode. Ia expression was found on cells with both low FSC and high

4. Discussion

In the present study we used immunocytochemistry and flow cytometry to examine the inflammatory infiltrate and MHC class II (Ia) antigen expression in the CNS of Lewis rats with CR-EAE induced by inoculation with guinea pig spinal cord and treatment with low dose CsA (see Polman et al., 1988; Pender et al., 1990) and of Lewis rats with acute EAE. In acute EAE and in the first episode of CR-EAE the infiltrate was composed mainly of CD4$^+$ T cells and macrophages. After recovery from acute EAE and in the first remission of CR-EAE the mean numbers of CD2$^+$ cells, TCR$\alpha\beta^+$ T cells, IL-2R$^+$ cells, CD4$^+$ T cells and CD8$^+$ cells were less than half of those in the preceding episode. Previous studies have shown that few T cells remain in the CNS of Lewis rats (McCombe et al., 1992) and mice (Zeine and Owens, 1993) that have recovered from acute EAE. To be lost from the spinal cord, cells may exit from the CNS or may die by apoptosis. We have described apoptosis of T lymphocytes in the spinal cord in acute MBP-EAE and suggested (Pender et al., 1991, 1992) that this apoptosis may contribute to the resolution of the CNS inflammation and possibly to resistance to re-induction of disease by re-inoculation (sec Willenhorg, 1979). FSC.

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Table 4
Numbers of macrophages, microglia and Ia$^+$ cells obtained from the spinal cord (cells $\times 10^6$/g)

	Total CD11b$^+$ cells (macrophages and microglia)		CD11b$^+$OX1^{high} (macrophages)		FSC^{high}OX1^{high} (lymphocytes and macrophages)		FSC^{low}OX1^{low} (microglia)		CD11b$^+$OX1^{high}/CD11b$^+$		Ia$^+$ cells	
	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*
Acute EAE												
Acute episode	1.76 ± 0.67	5	0.54 ± 0.10	3	2.75 ± 1.23	5	0.63 ± 0.24	5	32.4 ± 2.2	3	2.26 ± 0.91	5
After recovery	2.50 ± 1.84	4	0.50 ± 0.14	3	1.33 ± 0.72	3	0.74 ± 0.44	3	18.2 ± 4.9	3	2.00 ± 1.26	3
Acute vs. recovery	NS	NS	NS	NS	$P = 0.01$	NS						
CR-EAE												
First episode	1.84 ± 0.96	5	0.79 ± 0.32	2	2.74 ± 0.36	5	0.93 ± 0.46	5	51.5 ± 27.6	2	2.23 ± 0.70	5
First remission	1.75 ± 1.01	5	0.34 ± 0.15	4	1.07 ± 0.72	5	0.84 ± 0.49	5	20.9 ± 7.3	4	1.85 ± 1.08	5
vs. 1st episode	NS	NS	NS	NS	NS	NS						
Second episode	5.33 ± 1.76	5	2.17 ± 0.92	2	5.01 ± 2.29	5	2.18 ± 0.75	5	29.9 ± 8.5	2	6.32 ± 2.25	5
vs. 1st episode	$P^* < 0.05$	NS	NS	$P^* < 0.05$	NS	$P^* < 0.01$						
vs. 1st remission	$P^* < 0.01$	$P^* < 0.05$	$P^* < 0.05$	$P^* < 0.05$	NS	$P^* < 0.01$						
Third episode	3.72 ± 0.88	5	0.42 ± 0.13 ($P^* < 0.05$ vs. 2nd episode)	5	1.87 ± 0.81	5	2.22 ± 0.43	5	11.3 ± 2.6 ($P^* < 0.05$ vs. 1st episode)	5	4.04 ± 0.93	5
ANOVA (all CR-EAE)	$P = 0.0006$		$P = 0.009$		$P = 0.0008$		$P = 0.0007$		$P = 0.0009$	$P = 0.0004$		
Acute EAE vs. first episode CR-EAE	NS		NS		NS		NS		NS		NS	
Recovery from acute EAE vs. 1st remission CR-EAE	NS		NS		NS		NS		NS		NS	

Rats after recovery from acute EAE were compared with rats in the first remission of CR-EAE to look for effects of CsA treatment that might account for the development of relapses. We have hypothesized that treatment with CsA, which can inhibit activation-induced T cell apoptosis (Shi et al., 1989), prevents the apoptotic elimination of encephalitogenic T cells in the CNS (Pender, 1993). This might cause greater numbers of T lymphocytes to remain in the CNS of CsA treated rats in remission after the first episode of CR-EAE than in rats that have recovered from acute EAE; however, with the exception of TCR$\alpha\beta^+$ cells, which were more frequent in the rats recovered from acute EAE, there was no significant difference. Because only a minority of the T cells in the CNS in EAE are thought to be encephalitogenic (Smith and Waksman, 1969; Sedgwick et al., 1987) we cannot exclude the possibility that low dose CsA is blocking apoptosis of encephalitogenic T cells in the CNS. Low dose CsA could also inhibit activation-induced apoptosis of encephalitogenic T cells or their precursors in the thymus. Alternatively, low dose CsA treatment might cause relapses by selectively inhibiting downregulatory cells, such as the $CD4^+$ transforming growth factor-n-producing cells described by Karpus and Swanborg (1991).

To determine which cells are associated with relapses of EAE, we quantitated the infiltrating cells in the spinal cord in second and third episodes of CREAE and found increased numbers of T lymphocytes in the second episode of CR-EAE compared to the first remission, while in the third episode the number of T lymphocytes declined compared to that in the second episode. Polman et al. (1988) found T cells in the CNS in this model of CR-EAE 50 days after inoculation, but did not study rats throughout the course of CR-EAE. Immunocytochemical studies of CR-EAE in the guinea pig have demonstrated T cells in the spinal cord (Traugott et al., 1982; Antoniou et al., 1987a; Butter et al., 1989). In mice, immunocytochemical studies demonstrated $CD4^+$ T cells in the CNS in relapses of CR-EAE (Traugott et al., 1986; Baker et al., 1990) and radio-labelling has demonstrated passively transferred cells in the nervous system, especially in perivascular cuffs, in CR-EAE in SJL/J mice (Cross et al., 1990). These studies, together with the present study which clearly demonstrates an influx of $CD4^+$ T cells into the CNS in the second episode of CR-EAE, suggest that $CD4^+$ T cells, the effector cells in acute EAE (Pettinelli and McFarlin, 1981; Zamvil et al., 1985), continue to be important in relapses of CR-EAE.

More $CD4^+$ T cells than $CD8^+$ cells were present in both acute EAE and the first episode of CR-EAE and the ratio did not change significantly throughout the course of disease. Matsumoto et al. (1993) found significantly more $CD4^+$TCR$\alpha\beta^+$ cells than $CD8^+$ cells in extracts from the spinal cord of rats with MBP-EAE. Polman et al. (1988), using immunocytochemistry, found approximately equal numbers of $CD4^+$ and $CD8^+$ cells in rats with CR-EAE. The role of $CD8^+$ cells in EAE is unclear, but effector and immunoregulatory roles have been suggested. Mice which are genetically deficient in $CD8^+$ cells have a milder initial episode of disease and develop spontaneous relapses of EAE (Koh et al., 1992). Mice depleted of $CD8^+$ cells with antibody lose resistance to a second induction of EAE (Jiang et al., 1992), although earlier studies in mice and rats had shown no effect of anti-CD8 antibodies on the course of EAE (Waldor et al., 1985; Sedgwick et al., 1988). In the present study, the ratio of IL-$2R^+$ cells to $CD2^+$ cells did not change significantly throughout the course of CR-EAE. IL-2R expression occurs early and transiently after T cell activation (Smith, 1988). The IL-2R population may include the encephalitogenic T cells (Sedgwick et al., 1987).

In acute EAE and throughout the course of CR-EAE, TCR$\alpha\beta^+$ cells constituted about 65% of $CD2^+$ cells. $CD2^+$ TCR$\alpha\beta^+$cells in the CNS in EAE could include $\gamma\delta$ T cells, natural killer (NK) cells or cells which have down-regulated the expression of TCR$\alpha\beta$. $\gamma\delta$ T cells have been found in the CNS in multiple sclerosis (Selmaj et al., 1991) and may be cytotoxic towards oligodendrocytes (Freedman et al., 1991). NK cells are large granular lymphocytes which might be predicted to have higher FSC and SSC than small lymphocytes. As our $CD2^+$ population included cells of higher FSC and SSC than the TCR$\alpha\beta^+$ cells we suggest that some of the $CD2^+$ TCR$\alpha\beta^-$ cells may be NK cells.

We studied the expression by $CD2^+$ cells and TCR$\alpha\beta^+$ cells of CD45RC, the high molecular mass form of the leukocyte common antigen. Our $CD45RC^+$ and $CD45RC^-$ cells correspond to

the cells which are now described as $CD45RC^{high}$ and $CD45RC^{low}$ by Mason and co-workers (Powric and Mason, 1990; Fowell et al., 1991) but which were initially described as $CD45RC^{+}$ and $CD45RC^{-}$ (Powrie and Mason, 1989). $CD45RC^{-}$ T cells include memory cells (defined as having a strong response to recall antigens) (Ericsson et al., 1991) while $CD45RC^{+}$ T cells include naive cells and probably some memory cells (Mason and Powrie 1990). CD45RC is lost after cell activation and cell division (Powrie and Mason, 1989, 1990; Deans et al., 1992), although it may be regained as a cell proceeds through the cell cycle (Bell and Sparshott, 1990; Yang and Bell, 1992). CD45RC expression has also been used to distinguish T cells with Th1-like or Th2-like functions. In the rat, $CD45RC^{+}CD4^{+}$ T cells produce IL-2 and act in graft-versus-host reactions (Powrie and Mason, 1988, 1989, 1990) and can be responsible for autoimmunity (Fowell et al., 1991) while some $CD45RC^{-}$ T cells are responsible for B cell help (Powrie and Mason, 1989) or can downregulate autoimmune disease (Fowell and Mason, 1993).

It is not clear whether CD45RC expression by T lymphocytes in the target organ in autoimmune disease is related to the activation status or functional capacity. In rats with acute EAE induced by inoculation with whole CNS tissue or MBP, the majority of the T cells in the spinal cord are $CD4^{+}$CD45RC (Jensen et al., 1992; McCombe et al., 1992). In mice with acute EAE induced by the passive transfer of labelled, MBP-sensitized lymph node cells, the labelled cells in the CNS are $CD45RB^{low}$ (equivalent to our $CD45RC^{-}$) (Zeine and Owens, 1992). In the present study, at all stages of CR-EAE the majority of $CD2^{+}$ T cells and $TCR\alpha\beta^{+}$ T cells are $CD45RC^{-}$. It is likely that the predominance of $CD45RC^{-}$ T lymphocytes in the CNS in CR-EAE indicates that most of the infiltrating T cells have been activated. Activated encephalitogenic T cells and cells of other specificities can enter the normal CNS (Wekerle et al., 1986; Hickey et al., 1991) and the CNS in EAE (Ludowyk et al., 1992).

To study B cells we used the OX33 antibody which labels a form of the leukocyte common antigen found on B cells but not on plasma cells (Pilarski and Jensen, 1992) and the OX12 antibody which binds to kappa chains. The ratio of $CD4^{+}$ T cells to B cells was less in the second and third episodes of CR-EAE than in the first episode. In a previous morphological study we found that plasma cells were prominent in the spinal cord in the later stages of this form of CR-EAE (Pender et al., 1990). Immunocytochemical studies have demonstrated many plasma cells and B cells in the CNS of guinea pigs with CR-EAE (Grundke-Igbal et al., 1980; Traugott et al., 1982; Bernheimer et al., 1988). As B cells can differentiate into plasma cells in target organs (Lee et al., 1992), antibody production in the CNS may contribute to the pathogenesis of CR-EAE, although circulating antibody also has a role (Linington and Lassmann, 1987; Linington et al., 1988; Gautam et al., 1991). Involvement of antibody could contribute to the prominent demyelination which occurs in the third episode of CR-EAE and in chronic persistent EAE (Pender et al., 1990). B cells might also act as antigen-presenting cells in the CNS.

CD11b expression was used to identify macrophages and microglia. With immunocytochemistry, $CD11b^{+}$ round cells (macrophages) were prominent at all stages of CR-EAE. $CD11b^{+}$ cells with dendritic morphology (microglia) were especially prominent in the later stages of CR-EAE, which could be due to increased expression of CD11b and/or an increase in the numbers of microglia. To assess numbers of microglia we used flow cytometry. Sedgwick and co-workers (1991) demonstrated that microglia can be isolated from the spinal cord of normal rats and rats with CNS inflammation and used a characteristically low expression of OX1 to identify microglia. In addition to low OX1 expression, we used FSC to identify microglia, because we consistently found a population of small cells in the $CD4^{+}$ and the $CD11b^{+}$ populations (which include microglia), but not in the $CD2^{+}$ or $TCR\alpha\beta^{+}$ populations. All cells extracted from the spinal cord fell either into a population of FSC^{low} $OX1^{low}$ cells or a population of FSC^{high} $OX1^{high}$ cells (cells of haematogenous origin). We conclude that the FSC^{low} $OX1^{low}$ cells represent microglia which are smaller than macrophages and lymphocytes (Milligan et al., 1991) and have a lower OX1 expression than haematogenously derived cells (Sedgwick et al., 1991). OX1 expression can be upregulated on microglia during CNS inflammation (Sedgwick et al., 1991) and there was some overlap of OX1 fluorescence between

our FSC^{high} $OX1^{high}$ and FSC^{low} $OX1^{low}$ populations. We confirmed our interpretation by double labelling and found $OX1^{low}$ $CD11b^{+}$ cells (microglia) and $OX1^{high}$ $CD11b^{+}$ cells (macrophages). The number of microglia and the proportion of $CD11b^{+}$ cells that are microglia increased in the later stages of CR-EAE. Sedgwick et al. (1991) suggested that microglia proliferate in inflammatory disease of the CNS, and Matsumoto et al. (1992a) found that microglia proliferate in acute EAE. We suggest that this proliferation continues in CR-EAE although we cannot exclude differentiation of infiltrating macrophages into microglia.

With immunocytochemistry we observed Ia expression on round cells and cells of dendritic morphology in acute EAE and the first episode of CR-EAE. In the later episodes of CR-EAE, prominent Ia expression on cells of dendritic morphology was observed with immunocytochemistry, and increased numbers of Ia^{+} cells, including FSC^{low} cells typical of microglia, were found with flow cytometry. In the CNS, Ia can be expressed on infiltrating lymphocytes, macrophages and microglia and has been widely described in acute EAE (Matsumoto et al., 1986; Vass et al., 1986; Konno et al., 1989; McCombe et al., 1992). In this model of CR-EAE, widespread Ia expression in the CNS was found 50 days after inoculation (Polman et al., 1988). In CR-EAE in the guinea pig, Antoniou et al. (1987b) found Ia expression on cells with dendritic morphology. Microglia can present antigen in vitro (Matsumoto et al., 1992b) and antigen presentation by Ia^{+} microglia could possibly play a role in the development of inflammation in CR-EAE. However, we observed widespread Ia expression on microglia throughout the spinal cord, including areas with little inflammation, which suggests that la expression by microglia does not necessarily lead to inflammatory infiltration. An alternative possibility is that, in EAE, microglial Ia expression occurs as a reaction to the disease. Widespread Ia expression occurs as a response to other conditions affecting the CNS such as ischaemia (Gehrmann et al., 1992), graft-versus-host disease (Kajiwara et al., 1991) and distant trauma (Gehrmann et al., 1991). Such reactive Ia expression in EAE could play a role in downregulating the immune attack as, in mice, interferon-gamma (IFN-γ), which induces Ia expression, can suppress EAE (Billiau et al., 1988; Voorthuis et al., 1990) and antibodies to IFN-γ can enhance EAE (Billiau et al., 1988), and strains of rat that have constitutive Ia expression by microglia are resistant to the development of EAE (Sedgwick et al., 1993). We have proposed one possible mechanism by which MHC class II expression could downregulate disease which is that antigen presentation by non-specialised CNS antigen-presenting cells, that do not produce the co-stimulatory signal which is necessary for T cell activation (Jenkins et al. 1988), may lead to activation-induced apoptosis of encephalitogenic T cells (Pender et al., 1992; Pender, 1993). In the early stages of this model, CsA may protect from such apoptosis (see above) but after the cessation of CsA, activation-induced apoptosis could contribute to the eventual resolution of CNS inflammation.

In conclusion, we have studied CR-EAE in the Lewis rat and shown that T cells are present at all stages of disease, but have suggested a role for B cells in later stages of disease. The majority of T cells were $CD45RC^{-}$, which probably indicates that these cells have been activated in the periphery or in the CNS. During the later episodes of CR-EAE there was a marked increase in Ia expression by microglia and increased numbers of microglia.

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