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Expression of CD45RC and Ia Antigen in the Spinal Cord in Acute Experimental Allergic Encephalomyelitis: An Immunocytochemical and Flow Cytometric Study

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Abstract

We performed immunocytochemical studies to analyze the inflammatory infiltrate and major histocompatibility complex class II (Ia) antigen expression in the spinal cord of Lewis rats with acute experimental allergic encephalomyelitis (EAE) induced by inoculation with myelin basic protein and adjuvants. Using antibodies to lymphocyte markers and other monoclonal antibodies we found that during clinical episodes the inflammatory infiltrate was chiefly composed of T lymphocytes and macrophages. The majority of cells in the inflammatory infiltrate were stained by the W3/25 antibody to CD4 and a proportion was stained by OX22 which labels the high molecular weight form of the leucocyte common antigen (CD45RC). CDB+ T cells were sparse and B cells were not detected. There was minimal staining with the OX39 antibody to the interleukin-2 receptor. Presumptive microglia, identified by their dendritic morphology, expressed Ia antigen during the clinical episodes and after recovery. The prominence of Ia antigen expression after recovery could indicate that this la expression was associated with downregulation of the encephalitogenic immune response. We also performed flow cytometry studies on cells extracted from the spinal cord of rats before and during attacks of EAE. With flow cytometry, we found that in established disease a mean of 83(SD, 23)% of CD2+ cells were CD4+, and a mean of 27(SD, 12)% of CD2+ cells were CD45RC+. In rats sampled on the first day of signs, a mean of 43(SD, 22)% of CD2+ cells were CD45RC+. In the cells extracted from the spinal cord of rats with established disease a mean of 47(SD, 32)% of macrophages were CD45RC+. Our study has combined an immunocytochemical assessment of tissue sections with quantitative flow cytometry assessment of cells extracted from the spinal cord of rats with acute EAE. We have shown that the majority of T lymphocytes in the spinal cord are CD45RC$^-$. We have also found prominent Ia expression on dendritic cells in acute EAE and after clinical recovery.

Keywords: experimental allergic encephalomyelitis; lymphocyte; microglia, myelin basic protein; immunocytochemistry; autoimmunity; Ia antigen; flow cytometry

Introduction

Experimental allergic encephalomyelitis (EAE) is an autoimmune demyelinating disease induced by inoculation of susceptible animals with central nervous system myelin (CNS) antigens and adjuvants. The pathology of EAE is dominated by inflammation and demyelination in the central and peripheral nervous systems (Raine 1984; Pender 1987). Immunocytochemical studies of different models of EAE have shown that the inflammatory infiltrate consists of macrophages and T lymphocytes. EAE induced by inoculation with myelin basic protein (MBP-EAE) is useful in the study of the pathogenesis of the EAE because the target antigen is well characterized. In the Lewis rat two previous immunocytochemical studies of actively induced MBPEAE (Hickey et al. 1983, 1985) have shown that the predominant inflammatory cells are CD4+ and express the major histocompatibility complex class II (Ia) antigen. In the rat, the monoclonal antibody OX22 labels an epitope found in exon 5 of CD45RC, a high molecular weight form of the leucocyte common antigen which is expressed on B cells and CD4 + and CD8 + lymphocytes (Spickett et al. 1983; Barclay et al. 1987; Thomas and Lefrancois 1988; Powrie and Mason 1990, 1991). OX22 labelling divides CD4+ T lymphocytes into two populations (Spickett et al.

1983; Powrie and Mason 1990; 1991). CD45RC+CD4+ T cells are the more potent producers of interleukin-2 (IL-2) and interferon-y (IFN-γ) (Mason and Powrie 1990), whereas the $CD45RC^{-}CD4^{+}$ subpopulation provides B cell help and produces interleukin-4 (Arthur and Mason 1986; McKnight et al. 1991). We have used OX22 and other monoclonal antibodies to study further the inflammatory infiltrate and Ia antigen expression in MBP-EAE in the Lewis rat, using immunocytochemistry to assess the location of cells in sections of spinal cord, and flow cytometry to study cells extracted from the whole spinal cord.

Materials and methods

Animals

Lewis rats (JC strain) aged 9-12 weeks were obtained from the animal breeding facility of The University of Queensland.

Myelin basic protein (MBP)

MBP was prepared from guinea-pig spinal cords, from which the roots had been removed, by the method of Deibler et al. (1972). The purity of the preparation was established by SDS-polyacrylamide gel electrophoresis.

Induction of EAE

MBP in 0.9% saline was emulsified in an equal volume of incomplete Freund's adjuvant containing 4 mg/ml *Mycobacterium butyricum.* Rats were inoculated with 0.1 ml emulsion in each hindfoot. The total dose of MBP was 50 µg rat.

Clinical assessment

Animals were examined daily from day 7 after inoculation. Weakness of the tail and limbs was assessed and graded on a scale of 0 (no weakness) to 4 (complete paralysis) for each region as previously described (Pender 1986). A total disability score was obtained by adding the scores for the three regions.

Controls

Uninoculated normal animals were used as controls.

Preparation of tissue for immunocytochemistry

In our initial studies, rats were perfused with Nakane's solution (McLean and Nakane 1974). These animals were dissected and, after further immersion in fixative, specimens were immersed overnight in sucrose and frozen for cryosectioning. In later experiments, rats were perfused with ice-cold saline, the spinal cords were removed rapidly by insufflation, the tissue was immediately frozen, and frozen sections of unfixed tissue were prepared. These sections were then fixed in acetone/ ethanol for 5-10 min and used immediately or stored at - 20°C for use up to 6 weeks later.

Antibodies

The following monoclonal antibodies were obtained from Serotec: R73 (a//3 T cell receptor), OX34 and OX54 (CD2), OX52 (T cells), OX19 (CD5), OX39 (CD25; IL-2 receptor [IL-2R]), OX22 (CD45RC), OX8 (CD8), OX33 (leukocyte common antigen on B cells), OX6 (Ia antigen), OX42 (type 3 complement receptor on macrophages and microglia) and W3/25 (CD4 on T cells and macrophages). Polyclonal antibody to glial fibrillary acidic protein (GFAP) was obtained from Dako and to MBP was obtained from advanced immunocytochemicals.

Isolation of inflammatory cells from spinal cords

Rats with clinical signs of MBP-EAE were perfused via the left ventricle with cold, sterile, isotonic saline supplemented with 10 IU/ml sodium heparin. Spinal cords were removed by insufflation, weighed, passed through a 200-mesh stainless steel sieve and suspended in Hank's balanced salt solution (Sigma). Inflammatory cells were isolated

by the method of Cohen et al. (1987). A two-step discontinuous Percoll (Pharmacia) gradient was prepared by adding 10 ml isotonic Percoll to the spinal cord suspension and underlaying this 1.04 g/ml layer with 5 ml 1.08 g/ml Percoll solution. After centrifugation at 1500 x *g* for 30 min at 4°C, inflammatory cells were collected from the 1.04-1.08 interface.

Immunostaining

In preliminary experiments, 5-µm sections were incubated with mouse monoclonal antibodies and then with biotinylated anti-mouse immunoglobulin and horseradish peroxidase-conjugated avidin according to the methods of Lassmann et al. (1986b). In later experiments, sections were incubated with primary mouse monoclonal antibodies and then with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts). In all cases, the sections were exposed to normal rabbit serum to block non-specific binding, and washed care-fully with phosphate-buffered saline between incubations. The sections were exposed to diaminobenzidine for 10-15 min and, if required, were counterstained with Mayer's haematoxylin. Sections were assessed on a Zeiss Axiophot microscope, and cells were regarded as positively stained if the cell outline was dark brown. In all cases, sections without primary antibody were used as controls. Indirect peroxidase staining of cells extracted from the spinal cord was performed on cells in suspension. After staining the cells were air-dried onto a slide for microscopic examination.

Immunofluorescent-labelling of leukocyte surface markers and flow cytometry

Aliquots of 100 of cell suspension (2 x 10^6 to 10'/ml) were incubated with 100 µl *(5* µg) monoclonal mouse anti-rat leukocyte primary antibody (Serotec) for 30 min at 4°C. Cells were washed 2-3 times in 1 ml cold phosphate-buffered saline (PBS), 1% FCS, 0.1% sodium azide at 600 X *g* for 5 min at 4°C. The secondary antibody was a rat IgG-adsorbed affinity purified $F(ab')_2$ rabbit anti-mouse IgG-R-Phycoerythrin conjugate (Serotec). Secondary antibody was diluted 1 in 20 in PBS, with or without 10% normal JC Lewis rats serum. 100 Al aliquots of cell suspensions were incubated with 100 secondary antibody solution for 30-60 min at 4°C in the dark. Cells were again washed as described above. For double-labelling experiments fluorescein-conjugated monoclonal antibodies (5 µg protein) (Serotec) was incubated with cells in an incu-bation volume of 200 µl for 30 min at 4°C in the dark. Cells were washed as above and resuspended in 0.3 ml PBS, 1% FCS, 0.1% sodium azide and stored on ice in the dark prior to flow cytometric analysis. Control cell samples were incubated with secondary antibody only. The number of dead cells in each sample was estimated by incubation with 50 µg/ml propidium iodide (Sigma) for 5 min on ice before analysis. Blocking stages with incubation with 10% normal rabbit serum (Dako) for 10 min at 4°C prior to incubation with the first mouse monoclonal antibody, and either 10 µg normal Quackenbush mouse serum for 10 min before the addition of fluroescein-conjugated monoclonal antibodies, were included in the labelling procedure for some experiments.

Flow cytometry

Immunofluorescence analysis was performed using a FACScan flow cytometer and LYSYS software both supplied by Becton Dickinson. Fluorescein isothiocyanate (FITC), R-phycoerythrin (PE) and propidium iodide (PI) were excited using the 488 nm line of an argon laser and emission was detected at 530 nm (FITC) and 585 nm (PE/PI). Compensation for the detection of unwanted fluorescent signals, due to the overlap of emission spectra of the fluorescent dyes, was set when two- and three-colour fluorescence was used. Fluorescence data were collected using logarithmic amplification on 10 000 cells (both viable and non-viable) excluding events below a forward light scatter threshold of 140. Percentages of positively-labelled cell subpopulations were calculated by subtracting the equivalent control values in all cases.

Results

Clinical observations

Rats developed clinical signs of EAE (weakness of the tail, hindlimbs and sometimes forelimbs) on days 11-14 after inoculation. Neurologic signs were present for 3-6 days, after which time the animals regained normal strength.

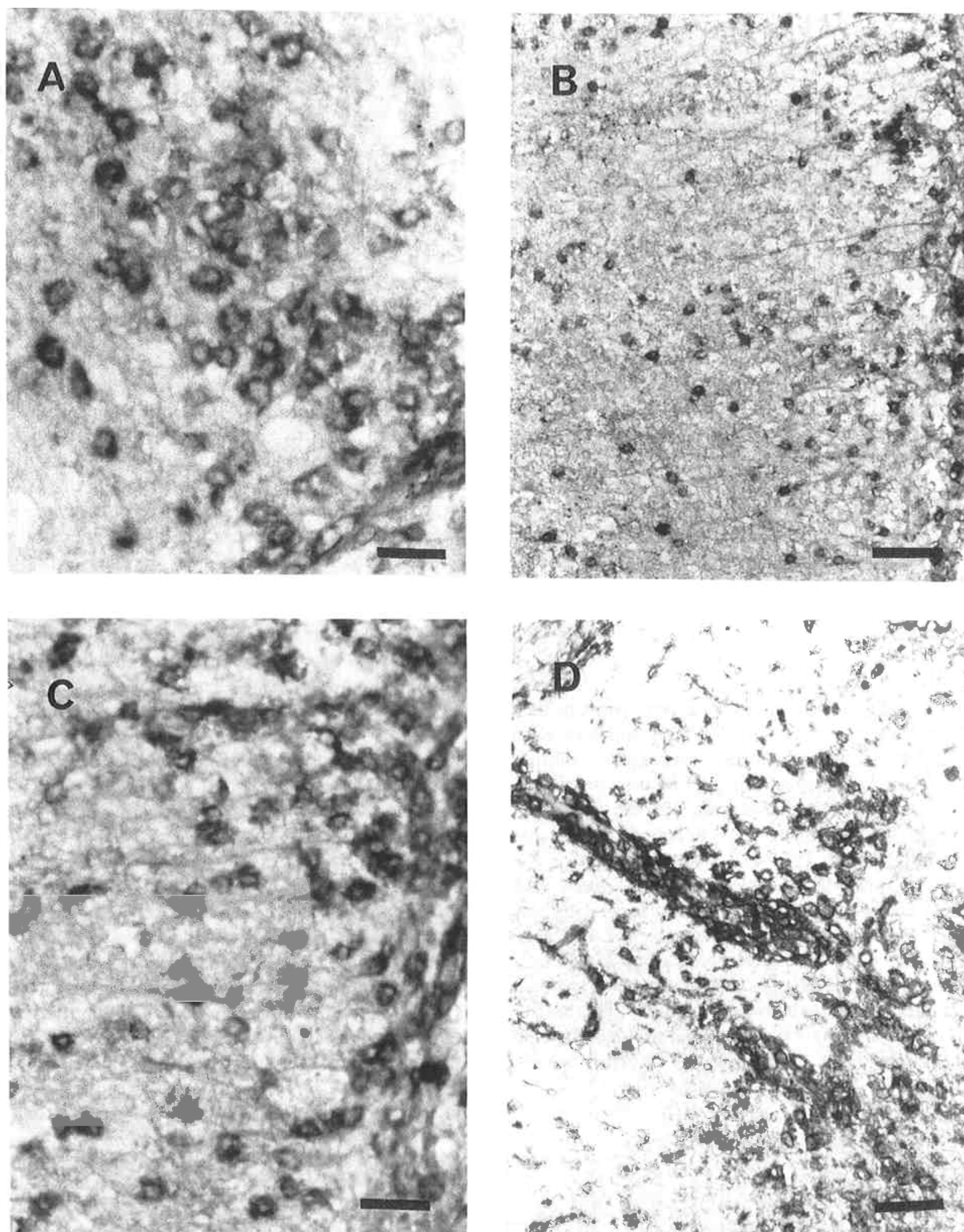

Fig. 1. Sections from the spinal cord of rats with neurological signs of MBP-EAE. Sections are stained with mouse monoclonal antibodies to cell surface antigens using the indirect peroxidase technique. A: section stained with OX34. There is a T cell infiltrate in the spinal cord. Bar = 25 μm. B: section stained with R73. There is a T cell infiltrate in the subpial region and in the white matter. Bar = 100 μm. C: section stained with OX22. There are many positively stained cells. Bar = 25 μm. D: section stained with OX42 which labels infiltrating macrophages and resident microglia. Many positively stained round cells and dendritic cells are present. Bar = 50 μm.

Preliminary trial of fixation and staining methods

Sections were prepared of tissues from animals perfused with Nakane's solution,

or from animals perfused with ice-cold saline. Tissue fixed with Nakane's solution gave inconsistent immunostaining results with high background staining and little staining with anti-lymphocyte antibodies. Tissue from the animals per-fused with ice-cold saline gave consistent immunostaining and low background staining, and these sections were used for the remainder of the study. More consistent results were obtained with the indirect peroxidase technique than with the avidin-biotin technique. The results of immunostaining of sections from rats with EAE were therefore obtained using indirect peroxidase staining of frozen sections from animals perfused with ice-cold saline.

Testing of different monoclonal antibodies

Lymph node. Monoclonal antibodies were tested on frozen sections of popliteal lymph nodes from a normal animal and an animal with EAE. All antibodies gave good staining. OX39, which labels the IL-2R on activated lymphocytes, was positive only on sections of the popliteal lymph node draining the inoculation site of the animal with EAE.

Nervous system. We compared different antibodies on spinal cord sections from EAE rats. These sections were shown by staining with haematoxylin and eosin to have a significant inflammatory infiltrate. The anti-lymphocyte antibodies R73 and OX34 were most effective and gave consistently good staining. OX52 and OX54 also showed good staining but OX19 (CD5) did not give strong staining of inflammatory infiltrates.

Immunostaining of lumbar spinal cord sections at different stages of EAE

The following results were obtained by immunostaining sections of lumbar spinal cord.

Controls. In two uninoculated control rats we found, as has been described by others (Hickey et al. 1985; Vass et al. 1986), that OX6 stained occasional round cells in the meninges and around blood vessels. OX42 stained presumptive microglia, identified by their dendritic morphology, in the grey and white matter of the spinal cord. W3/25 gave weak staining of similar dendritic cells. R73 stained very occasional round cells in the meninges.

Rats before neurological signs of EAE (days 9-12 after inoculation). In 1 rat sampled at day 9 and 1 rat sampled at day 10 the appearances were similar to those of controls. One rat at day 11 had an increased number of OX6-labelled cells in the meninges and one sampled at day 12 had increased numbers of both OX6^{+} and OX34^{+} cells in the meningeal region.

Rats with neurological signs of EAE (days 11-16 after inoculation). Three rats studied on the day of onset of neurological signs (at day 11 after inoculation) had an inflammatory infiltrate, which stained with antibodies to T cells and macrophages and to Ia, in the meningeal and perivascular regions. No la staining of parenchymal cells was observed.

Sections were taken from 6 rats with established neurological signs of EAE (at days 13-16 after inoculation). Of' these, 4 rats had tail weakness only and 2 had hindlimb and tail weakness. In all rats the staining pattern was similar. The T cell markers R73 and OX34 labelled large numbers of cells in the meningeal and subpial regions, in the white matter of the spinal cord and to a lesser extent in the grey matter. Many cells were located around blood vessels (Fig. 1). Cells la-belled with T lymphocyte markers were also present in the peripheral nervous system in the nerve roots. Cells labelled by the B cell marker, OX33, were not observed. There was strong staining of round cells in the meninges, around blood vessels and in the parenchyma with W3/25. W3/25 also labelled dendritic cells in the parenchyma. There was strong staining of round cells in perivascular regions, in submeningeal areas and diffusely in the white matter (Fig. 1) with the OX22 antibody. Comparison of adjacent sections indicated that cellular infiltrates that were labelled with R73 and OX34 also stained positively with W3/25 and OX22(CD45RC) (Fig. 2). OX8 stained only a small proportion of the cells. Very few

cells were stained with OX39. OX42 labelled round cells in meningeal regions, in the parenchyma and around blood vessels. This antibody also labelled resident dendritic cells throughout the spinal cord (Fig. 1). Some regions that were labelled with OX42 did not label with OX22 and vice versa (Fig. 3), indicating that OX22$^+$ cells that were not macrophages were present.

Strong staining was obtained with the OX6 antibody (Fig. 4). OX6$^+$ round cells were prominent in perivascular and meningeal regions and also were seen in the white matter and to some extent, grey matter. OX6$^+$ cells with dendritic morphology were also observed in white and grey matter in a similar distribution to OX42$^+$ dendritic cells.

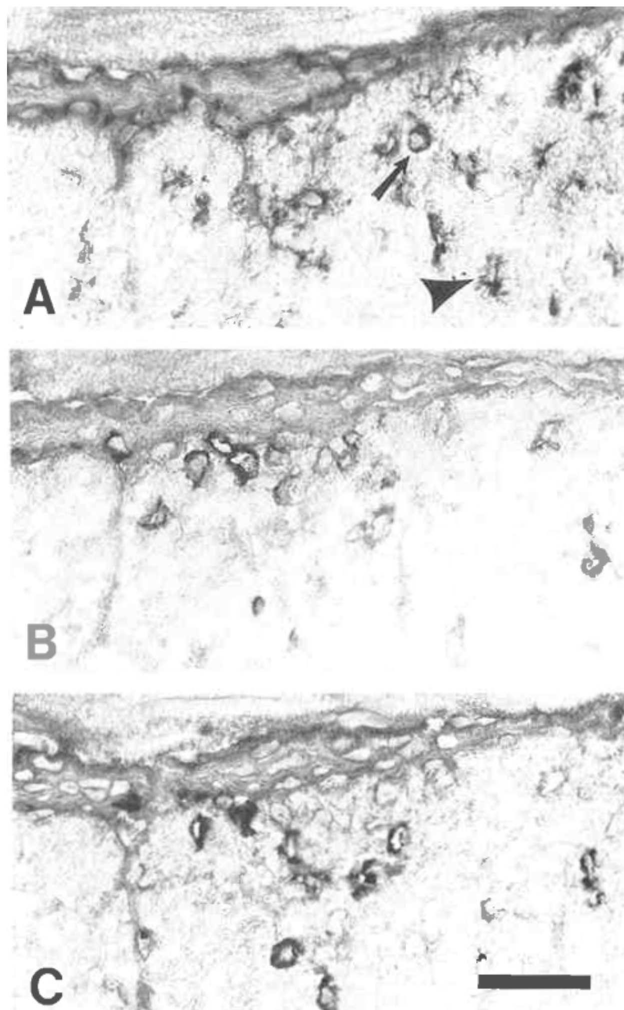

Fig. 2. Sections from the lumbar spinal cord of a rat with neurological signs of EAE. All sections are from the same block of tissue. A: labelled with W3/25. Positively stained round cells (arrow) are seen beneath the meninges. Some positively stained dendritic cells (arrowhead) are also seen. B: labelled with R73. Positively stained round cells are seen in the submeningeal region. C: labelled with OX22. Positively stained round cells are seen in a similar distribution to the cells in (B). These 3 sections show that cells in the same region are labelled with W3/25 (CD4), R73 (a/3 T cell receptor) and OX22 (CD45RC). Bar = 25 μm.

Rats studied after clinical recovery (days 15-33 after inoculation). Four rats were studied at this stage of disease. In all these rats the T cell infiltrate was reduced to a few small perivascular clusters. OX42$^+$ round cells were largely restricted to a perivascular distribution. OX42 labelling of dendritic cells in the parenchyma was increased compared to normal. Ia antigen was expressed on dendritic cells diffusely throughout the grey and white matter in rats studied at days 15, 16, 21 and 33 after inoculation (Fig. 4).

Extraction of cells from spinal cords

The mean yield of cells from rats on the first day of neurological signs (on days 11-13 after inoculation) was 1.2 X 10^6/g spinal cord (*n* = 6). The mean yield of cells from rats with established neurological signs of EAE on days 13 or 14 after inoculation was 4.5 x 10^6/g spinal cord *(n* = 16). The extracted cells were immunostained with indirect peroxidase labelling and examined on a slide. Many cells labelled with lymphocyte and macrophage markers. No cells labelled with anti GFAP, which labels astrocytes. Many cells labelled with anti MBP, but there were probably macrophages which had ingested myelin and expressed MBP, as macrophage labelling with anti MBP is a marker of demyelination (Prineas et al. 1989).

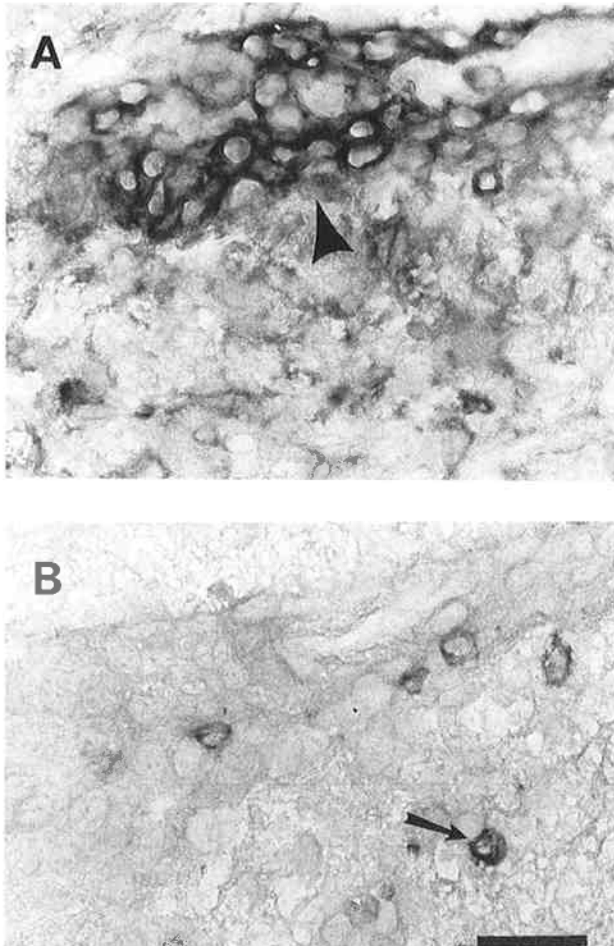

Fig. 3. Sections from the lumbar spinal cord of the same rat shown in Fig. 2. A: labelled with OX42. Perivascular cells (arrowhead) are densely labelled with OX42. In the parenchyma, the labelled cells have dendritic morphology. B: labelled with OX22. Few cells around the blood vessel are labelled. However, round cells labelled with OX22 are scattered through the parenchymal tissue (arrow). These sections show cells which are labelled with OX42 but not OX22 and vice versa. Bar = 25 μm.

Flow cytometry

The results of flow cytometry analysis of cells extracted from the spinal cord of rats with MBP-EA are shown in Table 1. In rats with established neurological signs of EAE, a mean of 57(SD, 19)% of cells were W3/25^+, a mean of 48(SD, 3)% were $OX34^+$, and a mean of 30(SD, 6)% were $OX22^+$. Because CD4 is expressed on macrophages and T lymphocytes, we per-formed double labelling to quantity $CD4^+$ T lymphocytes. In the cells extracted from the spinal cord, a mean of 83(SD, 23)% of $OX34^+$ cells were also $CD4^+$. Because of the possibility that OX22 may be expressed on macrophages as well as lymphocytes, we performed double labelling with OX22 and macrophage or T cell markers. OX22 labelled a mean of 27(SD, 12)% of $OX34^+$ cells and a mean of 47(SD, 32)% of $OX42^+$cells. As shown in Table 1, in cells obtained from

rats on the first day of EAE, a mean of 43(SD, 22)% of $CD2^+$ T cells were also OX22% and a mean of 90(SD, 36)% of $OX42^+$ cells were $OX22^+$.

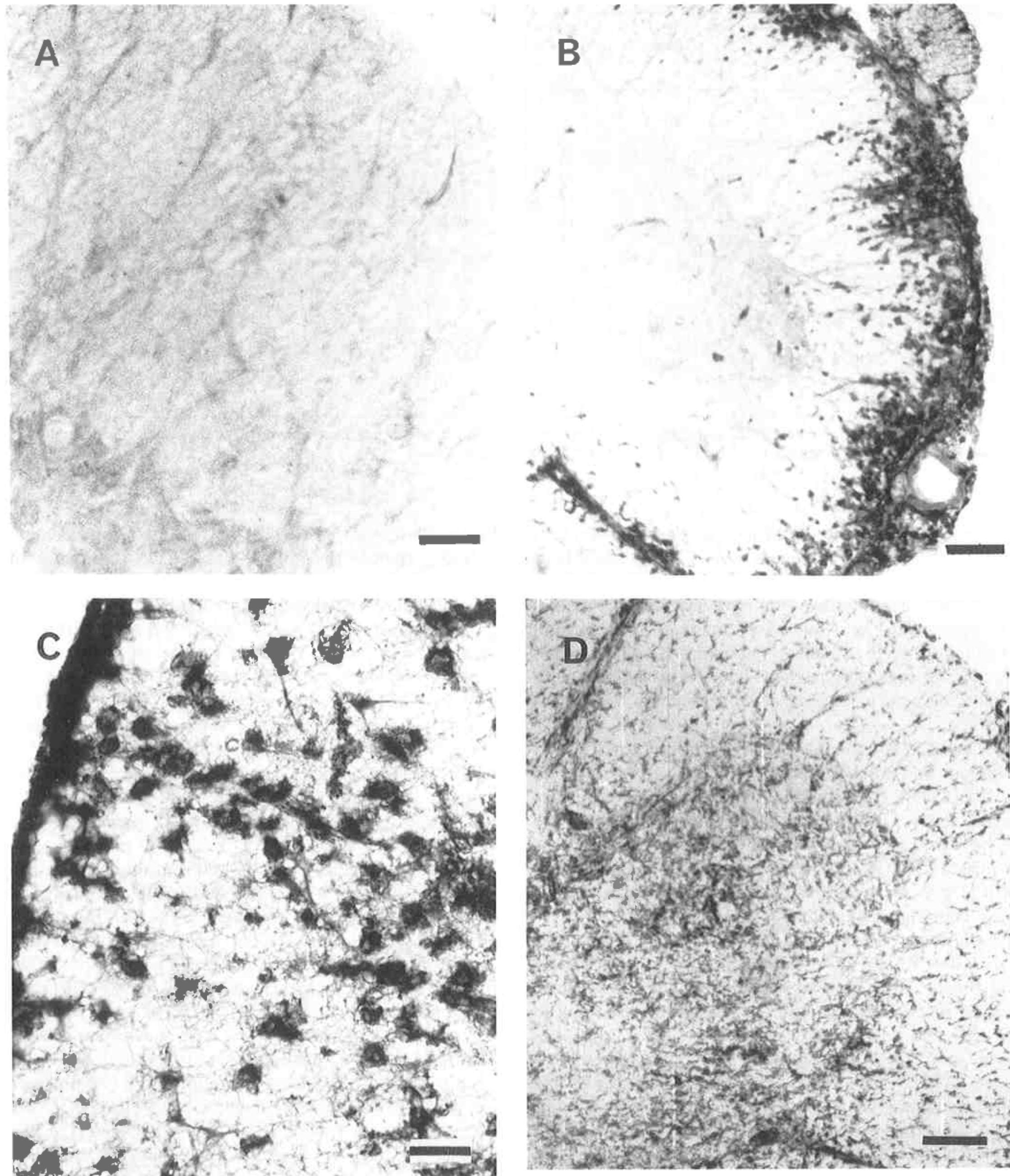

Fig. 4. Sections from the spinal cord of rats at different stages after inoculation with MBP. Each section is labelled with the OX6 (Ia) antibody using the indirect peroxidase technique. A: section from a rat with no neurological signs at day 11 after inoculation. In this field there is non-specific staining only. Bar = 25 μm. B: section from a rat on the first day of neurological signs of EAE. Positively stained cells are present in the meningeal and subpial regions. Bar = 100 μm. C: section from a rat on the second day of neurological signs. Many positively stained round cells and dendritic cells are present within the parenchyma. Bar = 25 μm. D: section from a rat on the first day after clinical recovery from MBP-EAE. Many positively stained cells are present throughout the cord. Most have dendritic morphology. Bar = 100 μm.

Discussion

In our immunocytochemical study on tissue from Lewis rats with acute MBP-EAE, we obtained the best staining using frozen tissue lightly fixed with acetone/ alcohol. Others have

also used fresh unfixed tissue in immunocytochemical studies of EAE (Traugott et al. 1982; Hickey et al. 1983; Sobel et al. 1984; Matsumoto and Fujiwara 1987). Using paraformaldehyde fixation, Lassmann et al. (1986a,b) were able to demonstrate lymphocytes and la expression in EAE. However, aldehyde fixation can reduce the availability of lymphocyte and macrophage antigens for immunostaining (Smit et al. 1974; Walker et al. 1984), and we chose to maximize the number of antigenic sites available for binding by avoiding aldehyde fixation.

In an earlier study of MBP-EAE Hickey et al. (1983) found few lymphocytes. We found that many of the infiltrating cells were T lymphocytes. This difference is most likely accounted for by our usage of newer anti-lymphocyte antibodies, especially R73 ($\alpha\beta$ T cells receptor) and OX34 (CD2). Our flow cytometric study of cells extracted from the spinal cord of rats with established neurological signs of MBP-EAE showed that 48% were $CD2^+$ (T cells or natural killer cells) and 24% were $OX42^+$ (macrophages or microglia). The remaining 28% may have been $OX42^-$ microglia. B cells were not detected in the present study using OX33 as a label. Hickey et al. (1983) found that in MBP-EAE a small percentage of Ia^+ cells had surface immunoglobulin and were thus typical of B cells. Small numbers of meningeal B cells have been described in acute EAE induced in guinea pigs by inoculation with bovine white matter (Traugott et al. 1982) or in mice by inoculation with isogeneic spinal cord (Traugott et al. 1985).

Our immunocytochemical studies showed that a high proportion of the infiltrating cells were $CD4^+$ while only a small proportion were $CD8^+$. With flow cytometry we confirmed that in established EAE, a mean of 57(SD, 19)% of the cells were labelled with CD4. Comparison of sections from the same region of inflammation suggested that many of these $CD4^+$ cells were $CD2^+$ and expressed the $\alpha\beta$ T cell receptor and were therefore $CD4^+$ T cells. With flow cytometry, we found that the percentage of $CD2^+$ lymphocytes was greater than the percentage bearing the $\alpha\beta$ T cell receptor. $CD2^+$ lymphocytes which do not bear the $\alpha\beta$ T cell receptor may be γδ T cells or natural killer cells. The presence of $CD4^+$ T cells was confirmed by flow cytometry where, in established disease, a mean of 83(SD, 23)% of $OX34^+$ cells were $CD4^+$. The $CD4^-$lymphocytes might include $CD8^+$ cells or γδ T cells. As $CD4^+$ lymphocytes can have cytotoxic (Sun and Wekerle 1986) or suppressor functions (Karpus and Swanborg 1989) as well as providing B cell help and mediating delayed-type hypersensitivity, staining with other markers is necessary to characterize these cells further.

TABLE 1

	Rats on 1st day of neurological signs (11-13 days after inoculation)			Rats with established neurological signs (12-14 days after inoculation)		
	Mean	SD	*n*	Mean	SD	*n*
Yield						
Cells/g	1.2 X 10^6		6	4.5 x 10^6		16
Percentage labelling with different markers[a]						
0X34	37	13	6	48	3	4
$R73^+$	21	2	3	32	19	3
$OX42^+$	16	11	6	24	10	3
$OX22^+$	24	14	6	30	6	5
W3/25	43	6	6	57	19	3
$OX34^+OX22^+$	16	12	5	13	6	4
(OX34 + OX22 $^+$)/OX34 +	43	22	6	27	12	4
$R73^+OX22^+$%	8	2.6	3	11	13	3
(R73 + OX22 +)/ R73 +	39	16	3	29	20	3
$OX34^+W3/25^+$	34	9	5	41	17	2
$(OX34^+W3/25^+)/OX34^+$	99	15	5	83	23	2
$OX42^+OX22^+$	12	6	6	9	5	3
$OX42^+OX22^+OX42^+$	90	36	6	47	32	3

[a] For the single markers, the percentage is that of the total population of cells extracted from the spinal cord. The ratios show the percentage of cells labelled with one marker which are also labelled with a second marker.

The OX22 monoclonal antibody binds to CD45RC (Powrie and Mason 1990, 1991) and can be used to divide $CD4^+$ T lymphocytes into two subsets (Spickett et al. 1983). Using

the OX22 monoclonal antibody we have found that many cells in this model of EAE are strongly $CD45RC^+$. The strong staining obtained with OX22 may occur because the leucocyte common antigen comprises approximately 10% of the surface of lymphocytes (Thomas and Lefrancois, 1988). OX22 labels CD45RC on $CD4^+$ and $CD8^+$ T lymphocytes and on B cells. There are no published studies of whether, in the rat, OX22 labels macrophages. As we found no B cells and only a small number of $CD8^+$ T lymphocytes in this study, we reasoned that the cells labelled with OX22 were $CD4^+$ T lymphocytes or possibly macrophages. With immunocytochemistry we found that some regions which were heavily labelled with OX22 were not labelled with the macrophage marker OX42 and vice versa (Fig. 4). We therefore concluded that at least some of the $CD45RC^+$ cells were T lymphocytes. With flow cytometric analysis of the extracted cells we found that a mean of 27(SD, 12)% of $CD2^+$ lymphocytes expressed high levels of CD45RC. We also found that a mean of 47(SD, 32)% of $OX42^+$ cells expressed high levels of CD45RC thus demonstrating that, in the rat, $OX42^+$ cells (macrophages or microglia) also can express CD45RC.

The $CD4^+$ $CD45RC^+$ T cell subset produces IL-2 and IFN-γ (Mason and Powrie 1990) and in this respect resembles the T helper 1 subset of mouse $CD4^+$ T cells, which can mediate delayed-type hypersensitivity and $CD4^+$ T-cell cytotoxicity (Erb et al. 1990). This subset also includes naive or unprimed T lymphocytes (Powrie and Mason 1988). In EAE, $CD45RC^+CD4^+$ T cells might be effector cells, as delayed-type hypersensitivity is commonly thought to occur in this disease. However, Sedgwick et al. (1987) have suggested a role for cytotoxic $CD4^+$ T cells in EAE. Our morphological studies suggest there may be apoptosis of oligodendrocytes in the nervous system in MBP-EAE (Pender et al. 1991). This suggests a possible role for $CD4^+$ cytotoxic T cells, as apoptosis is the mechanism of cytotoxic T-cell-induced target cell death (Pender et al. 1991). Using other methods, Hayosh et al. (1989) provided evidence that the effector cells in EAE are $CD45RC^+$ and Powrie and Mason (1990) have shown that autoreactive T cells are included in the $CD4^+OX45RC^+$ T cell population. However, Day and Mason (1990) found that an encephalitogenic T cell line was $CD45RC^-$. We found that the majority of the lymphocytes in the spinal cord were $CD45RC^-$. CD45RC is lost from $CD4^+$ T cells after activation by exposure to antigen (Powrie and Mason, 1989). The $CD45RC^-$ subset contains cells responsible for B cell help and cells able to regulate the $CD45RC^+$ population (Powrie and Mason 1990) and also cells which are responsible for memory (Fowell et al. 1991). Jensen et al (1989) found that the percentage of $CD4^+CD45RC^+$ cells was reduced in spinal cord compared to peripheral blood in rats with EAE induced by inoculation with guinea pig spinal cord. Clearly, further studies are required to determine whether the lack of expression of CD45RC on the majority of spinal cord T lymphocytes reflects activation of the cells or a functional capacity similar to mouse T helper 2 cells. Studies of cytokine production will help to answer this question.

In the present study, only a small proportion of the infiltrating T cells were activated as indicated by staining with the OX39 antibody to IL-2R. Sedgwick et al. (1987) demonstrated that only a small proportion of lymphocytes were $IL\text{-}2R^+$ in the spinal cord of rats with passively transferred MBP-EAE and suggested that this was because only CNS-specific T cells were likely to be activated in the CNS. It has been shown that only a small proportion of the cells in the CNS in EAE are likely to be MBP-specific (Cohen et al. 1987). Other possible explanations for the infrequency of $IL\text{-}2R^+$ cells in the CNS are that IL-2R may be ex-pressed for only a short period after T cell receptor stimulation (Smith 1988) or that, as we have recently suggested, MBP-specific T cells may be dying by apoptosis in the spinal cord and thereby being deleted (Pender et al. 1991, 1992).

We observed that Ia antigen, which is also expressed on activated T cells, was prominently expressed on infiltrating T cells and macrophages, and on resident dendritic cells (microglia) during established clinical disease. Polman et al. (1986) found la^+ lymphocytes in the spinal cord of rats with EAE induced by inoculation with spinal cord tissue. Lyman et al. (1989) identified la^+ lymphocytes in cells extracted from the spinal cord of rats with MBP-EAE. We found that the Ia expression on

dendritic cells persisted after clinical recovery, as observed by Matsumoto et al. (1986) and Konno et al. (1989) in EAE induced in the Lewis rat by inoculation with brain or spinal cord homogenate. Some of the increased Ia expression may be due to increased expression of Ia antigen on individual cells. However, there may also be proliferation of microglia in inflammatory CNS diseases (Sedgwick et al. 1991; Ohmori et al. 1992). As Ia expression is necessary for antigen presentation to $CD4^+$ T cells, it might be expected that Ia expression within the nervous system would enhance the development of EAE. However, the prominence of Ia expression on dendritic cells after clinical recovery raises the possibility that Ia expression has a role in the termination of the immune attack. Voorthuis et al. (1990) found that the intraventricular administration of IFN-γ, which increases Ia expression in the CNS, sup-presses EAE, and Konno et al. (1989) suggested that Ia expression may also assist with repair processes. Thus la expression in the later stages of EAE may be part of a protective response. Ia expression on non-professional antigen presenting cells may provide T cell receptor stimulation without providing the second signal, thus leading to anergy (Ohmori et al. 1992) or apoptosis of T cells (Pender et al., 1992).

In conclusion, this combined immunocytochemical and flow cytometry study of acute MBP-EAE found that $CD45RC^+$ cells were present in the inflammatory infiltrate but the majority of T cells were $CD45RC^-$. It also showed widespread microglial Ia expression which persists after clinical recovery.

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