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Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10.PL mice

(T cells/macrophages/microglia/astrocytes/multiple sclerosis)

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ABSTRACT **Experimental allergic encephalomyelitis (EAE) was generated in SJL and B10.PL mice by using the synthetic myelin basic protein peptides. Inflammation in brain and spinal cord preceded clinical signs of disease. Infiltrating lymphocytes were predominantly Lyt1$^+$ (CD5$^+$), L3T4$^+$ (CD4$^+$) T cells, until day 18. After that, F4/80$^+$ monocyte/macrophages outnumbered T cells. Ia$^+$ cells were microglia, macrophages, and endothelial cells, but Ia was not detectable on astrocytes in this EAE model. Ia$^+$ endothelial cells appeared later in the disease than Ia$^+$ microglia and macrophages, suggesting that antigen presentation at the blood–brain barrier is not initially responsible for inflammation. Cells staining for interferon γ, interleukin 2 (IL-2), and IL-2 receptors were more prominent than IL-4, IL-5, lymphotoxin (LT), and tumor necrosis factor α (TNF-α), which occurred transiently in the second week and were associated with fewer cells. TNF-α and LT were never seen in spinal cord, suggesting that these cytokines are not responsible for initiation of clinical disease. Few or no cells stained for IL-6, IL-1, or transforming growth factor β. Control animals injected with complete Freund's adjuvant in saline or control antigen demonstrated no inflammatory cell infiltration or cytokine production. Thus, our findings suggest a peptide-induced EAE model in which Th1 T-cell–macrophage interactions result in the disease process.**

Experimental allergic encephalomyelitis (EAE) is an autoimmune demyelinating disease of the central nervous system (CNS) of animals immunized with myelin basic protein (MBP). Adoptive transfer studies using antigen-sensitized lymph node cells or MBP-specific T-cell clones suggest that L3T4$^+$ (CD4$^+$) T cells, which use restricted T-cell receptor variable region genes, are specifically involved (1–4). The disease-inducing epitopes on MBP vary among mouse strains in which they are restricted by different major histocompatibility complex (MHC) class II molecules. We have previously demonstrated that a MBP peptide spanning residues 87–98 causes disease in SJL mice (H-2^s), while others have shown that EAE develops in B10.PL (H-2^u) animals in response to an acetylated N-terminal peptide (1–9NAc) (2). Nevertheless, the clinical pattern of disease in these strains is virtually identical and, moreover, is similar regardless of whether native MBP or peptides are used as the immunogen (5, 6).

Clinical and pathological changes in EAE resemble those in the human demyelinating disease multiple sclerosis (MS), for which EAE is proposed to be a model. *In situ* staining of MS lesions has shown a predominance of CD4$^+$ cells in the parenchyma adjacent to lesions and the presence of interferon γ (IFN-γ)-, interleukin 2 (IL-2)-, and IL-2 receptor (IL-2R)-positive cells at the lesion edge (7, 8). Creation of T-cell lines using MS cerebrospinal fluid T cells resulted in CD4$^+$ T cells producing IFN-γ and IL-2 (9). In mice, CD4$^+$ T cells have been thought to be classifiable into two subsets based on cytokine production and disease-related function. These are Th1 cells, which produce IL-2, IL-3, lymphotoxin (LT), and IFN-γ and induce delayed-type hypersensitivity and are active in Leishmaniasis and Brucellosis. IL-4-, IL-5-, and IL-6-producing Th2 cells seem to be important in resistance to *Nipostrongylus brasiliensus* by inducing immunoglobulin production (10). IL-10-producing Th2 cells appear to be able to inhibit proliferation and cytokine production of Th1 cells (11, 12). We have previously shown that encephalitogenic T cells produce cytokines of the Th1 subset *in vitro* (4). The predominance of IFN-γ and IL-2 early in the disease process suggests that Th1 cells, possibly in the absence of Th2 regulation, may be involved in this EAE model.

MATERIALS AND METHODS

Mice. SJL/J (12- to 14-wk-old females) and B10.PL mice (7- to 11-wk-old females) were purchased from The Jackson Laboratory. Four- to six-week-old outbred Swiss–Webster mice were used for herpes simplex inflammatory controls.

Immunization. Mice were immunized subcutaneously in the tail base with murine MBP peptides (peptides 81–100 and 1–9NAc) or saline and complete Freund's adjuvant (CFA) followed by *Bordetella pertussis* or *pertussigen* as described (5, 6). The herpes simplex virus-injected mice were provided by the laboratory of Jack Stevens (University of California, Los Angeles) (13). Control animals were sacrificed on day 15.

Disease Severity. Clinical EAE was graded on a scale of 1–5 by established standard criteria (5) as follows: grade 0, no discernible disease; grade 1, flaccid tail; grade 2, moderate hind or front leg weakness; grade 3, severe hind or front leg weakness; grade 4, complete paralysis of limb(s); grade 5, moribund.

Antibodies. Monoclonal antibodies used were as follows: rat anti-mouse Lyt1 (CD5), Lyt2 (CD8), L3T4 (CD4) (Becton

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Abbreviations: EAE, experimental allergic encephalomyelitis; IL, interleukin; IFN-γ, interferon γ; IL-2R, IL-2 receptor; LT, lymphotoxin; TNF-α, tumor necrosis factor α; MS, multiple sclerosis; TGF-β, transforming growth factor type β; MBP, myelin basic protein; CNS, central nervous system; MHC, major histocompatibility complex; CFA, complete Freund's adjuvant; PMN, polymorphonuclear leukocyte.

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Dickinson), Ia (Boehringer Mannheim), IL-2R (7D4; ref. 14), IL-2 (S4B6; ref. 15), IFN-γ (XMG1.2; ref. 16), macrophage (F4/80; ref. 17), IL-4 (11B11; ref. 18), IL-5 (TRFK5; ref. 19), and IL-6 (MP5-17D2; gift of John Abrams, DNAX, Palo Alto, CA). Polyclonal antibodies used were as follows: rabbit anti-mouse tumor necrosis factor α (TNF-α) (Genzyme), IL-1α (Advanced Magnetics, Cambridge, MA), anti-human LT (TNF-β) (Endogen, Boston), transforming growth factor type β (TGF-β) (R&D Systems, Minneapolis), and factor VIII (DAKO, Carpenteria, CA). Astrocytes were identified by rabbit anti-glial fibrillary acidic protein (DAKO). Optimal antibody concentrations were determined on mitogen-stimulated SJL/J and B10.PL spleen cell preparations. The anti-human LT and TGF-β cross-reacted with mouse LT- and TGF-β-producing cells from stimulated mouse spleen. Negligible background was observed with control isotype-matched antibodies of irrelevant specificities.

Immunocytochemistry. Brains were bisected in the midsaggital plane; half the brain was snap frozen in liquid nitrogen and stored at −80°C and the other half was fixed in 10% buffered formalin. Entire spinal cords were snap frozen or formalin fixed. Methods for single or double staining have been described extensively (7, 20). In this study, streptavidin horseradish peroxidase/aminoethyl carbazol or diaminobenzidine, and alkaline phosphatase/Fast Red enzyme–substrate complexes were used. Mayer's hematoxylin was the counterstain used. Polymorphonuclear leukocytes (PMNs) were identified by their multisegmented nuclei and intense staining due to endogenous peroxidase activity. Luxol fast blue stain was used to stain for myelin by standard protocols (21). All major sites were examined in step sections and inflammatory cells and cells staining for cytokines were scored as either a percentage of total cells per mm^2 (meninges and blood vessels) or as an absolute number of positive cells per mm^2 (parenchyma).

RESULTS

Inflammation Precedes Clinical Signs in Peptide-Injected Mice: Ia$^+$ Cells, IL-2, and IFN-γ Are the First Signs of Disease. Table 1 shows the incidence, day of onset, and severity of disease in the MBP peptide and normal saline/CFA-immunized SJL and B10.PL mice analyzed in this study. In group 1 (Table 1), within the first week after immunization and before gross clinical signs were evident, lymphocytes, monocytes, and a few PMNs were present in the brains and spinal cords. Inflammatory cells were located in blood vessels and meninges in the spinal cord and cerebellum. Groups 2 and 4 (Table 1, early and late disease, respectively) demonstrated equal amounts of inflammation in brain gray and white matter as in spinal cord. Group 3 (possible refractory) had less inflammation in the spinal cord than in the brain. Groups 2–4 had the most inflammation in cerebellar white matter and subarachnoid space and less involvement of the cerebellum and hippocampus. Group 6, designated as very advanced disease, showed low numbers of inflammatory cells in both spinal cord and brain.

In group 1, the majority of inflammatory T cells present were CD4$^+$ (20–30% CD4$^+$ vs. ≤5% CD8$^+$). Ia$^+$ F4/80$^+$ monocytes were evident as perivascular infiltrates in the brain and spinal cord. Astrocytes and endothelial cells were Ia$^-$. The only lymphokine staining was diffuse IL-2 and IL-2R and IFN-γ in cerebellar white matter and superficial cortex perivascular cuffs as well as adjacent brain parenchyma. No other lymphokines were present in brain nor were any lymphokines found in spinal cord (Table 2).

In early (group 2) and late (group 4) disease, the CD4$^+$ T cells outnumbered CD8$^+$ T cells by 2- to 10-fold. Ia$^+$ macrophages and microglia, which stained with F4/80 in sequential sections, were present in the brain and spinal cord (Table 2; Fig. 1), while ≈10% of factor VIII$^+$ endothelial cells were Ia$^+$ by double staining (Fig. 2). Ia staining in spinal cord and brain increased with time postinjection. Morphologically identifiable astrocytes were Ia$^-$ and never stained for cytokines. There were more cells staining for IL-2R in brain and more cells staining for IFN-γ in spinal cord than had been seen before day 11. IL-2 and IL-2R$^+$ cells persisted in brain longer than in spinal cord. Usually associated with blood vessels and meninges, LT, IL-4, IL-5, and TNF-α were observed in fewer cells, in fewer animals, and for a shorter time period (days 11–15) in the disease than were IL-2 and IFN-γ. Comparing late disease (group 4) with very advanced disease (group 6), immune activity decreased with time; there were fewer macrophages and lymphocytes, and less MHC class II and IFN-γ expression (Table 2). Demyelination was evident and most prominent in spinal cords in group 2 animals but did not appear to correlate with inflammation or disease severity (data not shown).

In none of the six representative brains stained (from groups 2–6) did we observe IL-1 or TGF-β. IL-6 was seen associated with <10% of leukocyte infiltrates in blood vessels and meninges in only two (groups 1 and 3) of seven representative brains (groups 1–7). IL-6 staining of <5% of inflammatory cells was seen in one group 1 spinal cord.

Minimal Signs of Lymphocyte and Monocyte Activation Are Found in Control, Recovered, and Refractory Animals. The

Table 1. Clinical and pathological course of MBP peptide-induced EAE

Group (*n*)	Description	Day of onset (severity score)	Day of maximum disease severity (severity score)	Day of sacrifice (final severity score)	Inflammation	
					Spinal cord	Brain
1 (8)	Preclinical	— (0)	— (0)	5–11 (0)	+++	++
2 (3)	Early disease	9–10 (0.5)	11 (2)	11 (2.5)	++	++
3 (6)	Possible refractory	— (0)	— (0)	15–18 (0)	+	+++
4 (5)	Late disease	9–11 (0.5)	12–18 (2.5)	15–18 (2.5)	+++	+++
5 (4)	Refractory	— (0)	— (0)	28–82 (0)	±	+
6 (3)	Very advanced disease	6–15 (0.5)	12–82 (2)	23–82 (2)	+	+
7 (4)	Recovered	9–13 (0.5)	13–23 (2.0)	18–28 (0–0.5)	+++	++
8 (5)	Control	— (0)	— (0)	15 (0)	−	−

Day of onset, maximum disease, and sacrifice is the day postinjection. *n* is the number of mice per group. Groups 1–3 and 7 were entirely composed of SJL/J mice. Groups 4 and 5 have one B10.PL mouse each. Group 6 has two B10.PL mice. The remainder are SJL/J mice in groups 4–6. Group 8, normal saline/CFA controls. Inflammation was scored as follows: −, no inflammation—no cytokines; +, inflammation—meninges, blood vessel (lumen + wall); no perivascular or parenchymal infiltrates; ++, inflammation—meninges, blood vessel (lumen + wall); a few perivascular cells in Virchow Robin space; parenchymal infiltrates (<5/mm^2); +++, inflammation—meninges, blood vessel, perivascular cuffing; deep parenchymal infiltrates (10–30/mm^2). Percentage total cells in areas of inflammation: +, 1–5% lymphocytes and 0% monocytes; ++, 10–30% lymphocytes and 1–10% monocytes; +++, 30–50% lymphocytes and 10–30% monocytes. A minimum of 200 total cells were scored for percent.

Table 2. Inflammatory cell subsets and cytokines in MBP peptide-induced EAE

	Group						
	1 (preclinical)	2 (early disease)	3 (possible refractory)	4 (late disease)	5 (refractory)	6 (very advanced disease)	7 (recovered)
Brain							
Lyt1 (CD5)	++	++	++	+++	+	+	++
Lyt2 (CD8)	±	−	+	+	−	−	−
L3T4 (CD4)	++	++	++	+++	+	+	++
F4/80 (M)	+	+	++++	+++	++	++	+
Ia	+*†‡	+*†‡	++++*†‡	+++*†‡	++†‡	±*	±*†‡
IFN-γ	+*†‡	±*†	±*†‡	±*†	−	−	−
IL-2	±*†‡	±*†	±*†‡	±*	±*	±*†‡	−
IL-2R	±*†‡	+*†	±*†‡	±*†‡	±*	±*†‡	−
LT	−	−	±*	−	−	−	−
TNF-α	−	−	±*†	±*	−	−	−
IL-4	−	−	−	±*†	−	−	−
IL-5	−	−	−	±†	−	−	−
Spinal cord							
Lyt1	+++	++	±	+++	±	+	+++
Lyt2	+	+	−	±	−	−	−
L3T4	+++	++	±	+++	±	+	+++
F4/80	+	+	+	++	±	+	+
Ia	++†	++*†‡	++*†‡	+++*†‡	+*‡	−	+
IFN-γ	−	++*†	+†‡	±*†‡	−	−	−
IL-2	±†	+*†	±†‡	−	−	−	−
IL-2R	±†	±*‡	±‡	±*†‡	−	−	−
LT	−	−	−	−	−	−	−
TNF-α	−	−	−	−	−	−	−
IL-4	−	−	±*†	−	−	−	−
IL-5	−	+†‡	±*	−	−	−	−

−, No inflammatory cells, no cytokines; ±, ≤5% inflammatory cells; +, 5–15% inflammatory cells; ++, 20–30% inflammatory cells; +++, 40–50% inflammatory cells; ++++, 60–80% inflammatory cells. Saline/CFA control, all lymphokines negative, <2% lymphocytes, 5% ± 2% monocytes (M). A minimum of 200 total cells were scored for percent.
*Blood vessel-associated cells.
†Meningeal infiltrates.
‡Parenchymal infiltrates.

animals in group 7 (recovered) had more inflammation in spinal cord than brain, consisting of $CD4^+$ T cells and Ia^+ macrophages and endothelial cells. There were more Ia^+ endothelial cells than Ia^+ macrophages. No cytokines were found. The inflammation in brain was greater than in spinal cord in refractory animals (group 5) consisting of Ia^+ macrophages, IL-2^+, and IL-$2R^+$ cells (Table 2). These animals had very few $CD4^+$ T cells, few Ia^+ $F4/80^+$ macrophages, and no cytokines in spinal cord. The Ia^+ cells in spinal cord were endothelial cells. B10.PL and SJL mice showed similar clinical and histo- and immunochemical findings. Saline/CFA control animals had no inflammation or cytokine production. The herpes simplex virus-injected animals had a few B cells and macrophages but no T cells or cytokines (data not shown).

DISCUSSION

The predominant phenotype of EAE-inducing T cells in mice is $Lyt1^+$, $L3T4^+$, $Lyt2^-$ ($CD5^+$, $CD4^+$, $CD8^-$) as ascertained by adoptive T-cell transfer and monoclonal antibody depletion studies *in vitro* and *in vivo* (22–24). The responses to MBP and disease induction are MHC class II (Ia) restricted and mediated. Anti-Ia and anti-CD4 antibodies inhibit disease induction and progression (25, 26). Cells such as macrophages, microglia, astrocytes, and endothelial cells can be induced to express Ia *in vitro* in response to IFN-γ and to present MBP to T cells. Thus, there has been interest in determining the role of these cells and MHC class II molecules plus peptides and Ia induction *in vivo* by IFN-γ in EAE. The expression of Ia on astrocytes has been linked to disease susceptibility in some rat strains (27), while endothelial cells from EAE-susceptible guinea pigs have been shown not only to express Ia *in situ* but to present MBP to T cells activated to the same MBP *in vivo* (28). The specific cells that present pathogenic peptides *in vivo* are not known. Here, we have conducted a histochemical analysis of the pathology of disease induction by MHC class II-restricted and specific MBP peptides in an effort to compare the disease process to other EAE animal models and to the human disease MS. In addition, we have tried to put the inflammatory cells into the context of $CD4^+$ T-cell functional subsets proposed by Mosmann and colleagues (10).

As earlier investigators showed in EAE induced by cell transfer or purified MBP or even spinal cord homogenates, we have shown with MHC-restricted peptides that $CD4^+$ T cells predominate in the areas of inflammation. Inflammation in this model, as in other models, occurs earlier than clinical signs and disease onset may not correlate with the severity of demyelination (2, 3, 6, 29). Refractory animals (group 5) have a few $CD4^+$ and no $CD8^+$ T cells, very few Ia^+ macrophages, and minimal or no cytokine production in brain and spinal cord. This argues against $CD8^+$ suppressor cells mediating the suppression in this model even though $CD8^+$ cells can and do protect animals from EAE in other models (30). The absence of cytokines in recovered animals (group 7) suggests that down regulation of cytokines is associated with clinical recovery. In our studies, while PMNs appeared within the first week, they were only present in brains of animals with stage 0–2 disease. Thus, we presume that these cells do not cause or contribute to disease in peptide-induced EAE. Other reports, using adoptively transferred guinea pig MBP-sensitized lymph node cells or high doses of MBP in mouse EAE, have correlated inflammatory PMNs with severity of

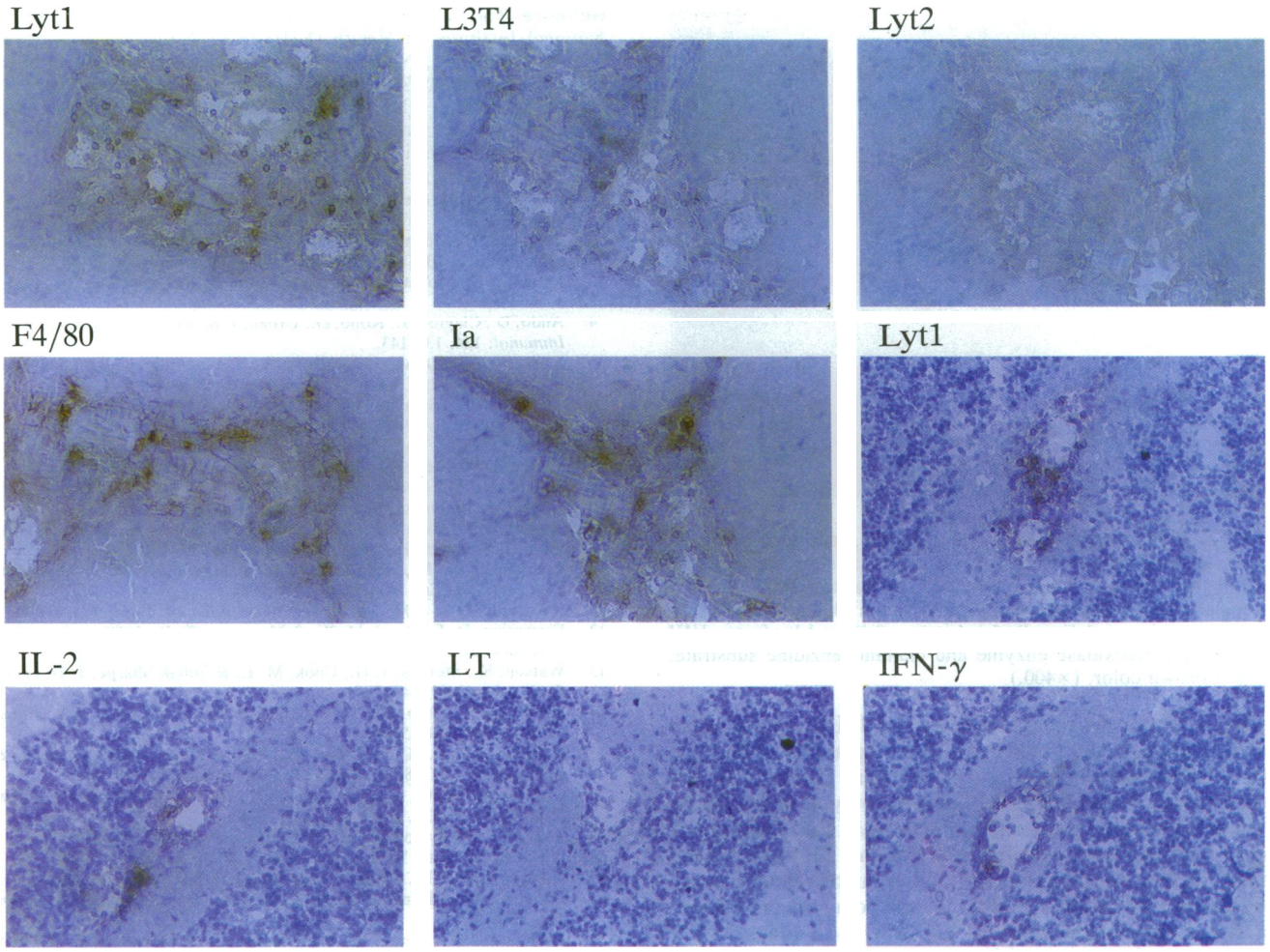

FIG. 1. Lyt1 (*Top Left*), L3T4, Lyt2, F4/80, and Ia are shown from an SJL/J mouse with stage 1 disease, sacrificed at day 11 (group 2). Inflammation is in the hippocampal fissure and meninges. Lyt1 (*Middle Right*), IL-2, LT, and IFN-γ are shown from an SJL/J mouse with stage 2 disease, sacrificed at day 15 (group 4). Inflammation is in the white matter parenchyma of the cerebellum. No primary or control primary antibody control followed by anti-secondary antibody were negative (data not shown) and resembled negative LT shown above in this figure. (×400.)

disease (3). Use of restricted antigenic epitopes of MBP may diminish PMN involvement.

Our studies support observations that Ia^+ cells are present in the greatest numbers in spinal cord prior to clinical disease and persist in brain to advanced disease. Early Ia^+ cells in brain and spinal cord were primarily microglia with inflammatory macrophages entering later and expressing Ia. Similar to previous reports, we saw no evidence of Ia on astrocytes (29). Endothelial cells developed Ia only after inflammation and disease were well established. In fact, most Ia^+ cells in long-standing or severe disease and refractory animals were endothelial cells. These data are consistent with Sakai *et al.* (31), who described Ia^+ endothelial cells in chronic relapsing EAE. Ia^+ cells were reduced in recovered animals in this study. The differences between our data and those from other laboratories on astrocyte expression of Ia molecules are most likely explained by our use of small antigenic peptides of murine MBP as opposed to the whole protein or spinal cord homogenate. Whole MBP and spinal cord contain multiple antigenic determinants that may be required for Ia induction on astrocytes. We conclude that peptide-induced EAE may be representative of CNS inflammation, which follows restricted immune system activation by a single cross-reactive pathogenic determinant. Astrocyte involvement may be a secondary event in localized CNS inflammation. In this model, T cells and Ia^+ macrophages appear to cross the blood–brain barrier prior to Ia expression on endothelial cells. Our data support late Ia expression on endothelial cells and predominance in the brain and suggest a role for the endothelium as a site of antigen presentation in chronically diseased animals. Microglia and macrophages may serve a similar function earlier in acute disease.

In the brain sections examined, cytokines were associated only with microglia and inflammatory cells and not astrocytes, in spite of the fact that astrocytes stain with anti-TNF-α antibody in MS brain and have been shown to secrete cytokines *in vitro* (20, 32). Cells staining for IFN-γ, IL-2, and IL-2R were in greater numbers and appeared earlier in brain than in spinal cord. We have previously shown that IFN-γ-, IL-2-, and IL-2R-bearing cells occurred at the leading edge of MS plaque formation (7, 8). Indeed, IFN-γ treatment of MS patients aggravates the disease by increasing exacerbations and enhancing DR expression on macrophages (33). The absence of IFN-γ in recovered and refractory animals suggests that IFN-γ may play a role in the induction and disease progression in this animal model. This is in contrast to the primary inflammatory demyelinating control model induced by herpes simplex (34, 35) where there are no T cells and no Ia^+ macrophages or cytokines. Our inability to consistently detect IL-6 in peptide-induced EAE again is in contrast to findings by Gijbels *et al.* (36); this may relate to the antigen used or to increased sensitivity of the bioassay.

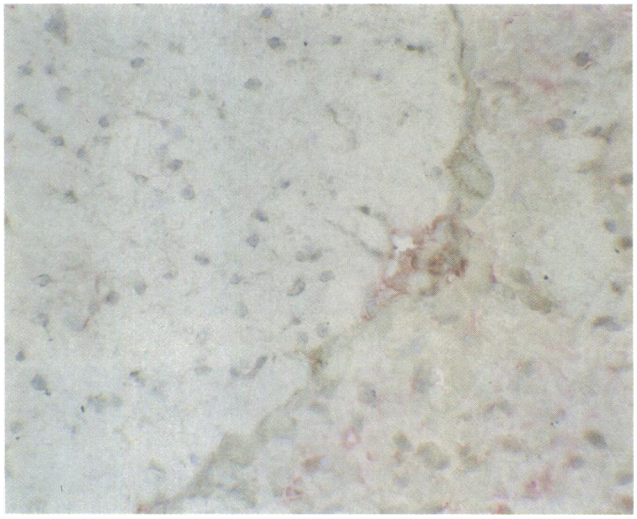

FIG. 2. SJL/J mouse with stage 2 disease, sacrificed at day 15 (group 4). Staining of blood vessels with perivascular cuffs of inflammatory cells in white matter of cerebellum. MHC class II molecules were stained and visualized with the alkaline phosphatase enzyme complex and Fast Red substrate, followed by factor VIII staining with peroxidase enzyme and diaminobenzidine substrate, giving a brown color. (×400.)

We have demonstrated that IFN-γ and IL-2 are produced by encephalitogenic T cells (4), implying a Th1 phenotype (10). We detected small amounts of IL-4 and IL-5 in brain and spinal cord; LT and TNF were present only in brain. These cytokines appeared primarily in the interval between days 11 and 15 postinjection, possibly implying that Th2 cells played no role in disease induction and only a minor role in disease progression. Th1 cells produce LT and some encephalitogenic T-cell clones may produce LT (37). EAE induction by passive transfer can be blocked by using a monoclonal antibody to LT/TNF-α (38). Low levels of production of LT in this EAE model in the second week would argue against, although not rule out, extensive T-cell cytolysis or myelin damage by such a mechanism. The Th1 profile of high IL-2 and IFN-γ but low LT *in vivo* also argues for differences in T-cell lymphokine patterns and/or differences in subsets of Th1 cells *in vivo* compared to *in vitro*. It is also possible that disease-inducing T-cell clones are probably not fully representative of the whole T-cell response to MBP peptide. Low levels of TNF-α are also consistent with the paucity or absence of PMN infiltration and hemorrhage known to be induced by TNF-α and prominent in high dose MBP-induced EAE (3). Since Th2 cells would negatively regulate Th1 cells, their apparent absence as the disease progresses may be a significant factor in this model.

In summary, there are some similarities in the pathology of the EAE induced by MBP peptide with other EAE models and even with MS. There is an association of disease with cells that have the phenotype and may function like Th1 cells as well as with Ia^+ macrophages, microglia, and endothelial cells. IFN-γ and IL-2 are important in induction and perpetuation of the disease. We do not believe that astrocytes contribute to the peptide-induced EAE model in that they do not produce cytokines or act as antigen presenting cells.

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