Acute Experimental Allergic Encephalomyelitis in SJL/J Mice Induced by a Synthetic Peptide of Myelin Proteolipid Protein

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Abstract. Clinical, histologic, and ultrastructural characteristics of acute experimental allergic encephalomyelitis (EAE) induced by sensitization with a synthetic peptide corresponding to mouse myelin proteolipid protein (PLP) residues 139–151 HCLGKWLGHPDKF were studied in SJL/J mice. Groups of mice were immunized with 20, 50, or 100 nmol of the peptide and were killed from seven to 28 days after sensitization or when they were moribund. Beginning on Day 9, the mice showed signs of EAE and the disease progressed rapidly to paralysis. Central nervous system (CNS) inflammation, edema, gliosis, and demyelination were found in all mice killed between Days 10 and 28 and white matter lesion areas correlated with clinical score at the time the mice were killed. Peripheral nerve roots and the cauda equina did not have lesions. Within the range studied, the severity of clincal or histologic disease was the same regardless of the PLP peptide dose. Two of ten mice immunized with 100 nmol and none of 14 mice given smaller doses of a synthetic peptide of mouse myelin basic protein (MBP) showed clinical EAE. These mice had small numbers of CNS lesions that were indistinguishable from those in PLP peptide-sensitized mice. These findings demonstrate that immunization of SJL/J mice with PLP peptide 139–151 produces a disease with the clinical and morphologic features of CNS tissue-, whole PLP-, whole MBP-, and MBP peptide-induced acute EAE. Thus, PLP is a major encephalitogen and immune reactions to epitopes of different myelin proteins may induce identical patterns of injury in the CNS.

Key Words: Demyelinating diseases; Experimental allergic encephalomyelitis; Multiple sclerosis; Myelin; Proteolipid protein.

INTRODUCTION

Experimental allergic encephalomyelitis (EAE) is an extensively studied central nervous system (CNS) disease model which shares features with human CNS inflammatory and demyelinating diseases (1, 2). Acute EAE can be induced in experimental animals by immunization with whole CNS tissue, myelin, or myelin components. Myelin basic protein (MBP) has been studied for many years as an antigen which induces EAE (3). In contrast, only in recent years has myelin proteolipid protein (PLP), the major structural protein of CNS myelin, been generally accepted

as encephalitogenic (4–13), even though it was initially recognized as inducing EAE in the 1950s (14).

One approach to understanding mechanisms of immune responses is to use peptides corresponding to known protein sequences as immunogens (15–18). Analyses of T cell epitopes have provided insight into molecular interactions involved in cellular immune reactions and the pathogenesis of CNS inflammatory diseases (19). Recent studies have identified peptides derived from MBP (20, 21) and PLP (22) that are encephalitogenic in inbred strains of mice. In SJL/J mice, encephalitogenic peptide sequences of both MBP (23–26) and PLP (27) have been identified, suggesting that T cell responses to epitopes of different myelin proteins may contribute to EAE induced by sensitization with whole CNS tissue or myelin in an inbred strain.

In this study we analyze the clinical and morphologic features of acute EAE induced with a defined, HPLC-purified synthetic encephalitogenic epitope of PLP in SJL/J mice. Since quantitative aspects are important in the initiation of immune reactions (28) and the degree of demyelination in EAE (29), mice were sensitized with various doses of the peptide. For comparison, other groups of SJL/J mice littermates were sensitized with equivalent doses of a synthetic encephalitogenic epitope of mouse MBP. The mice were killed at pre-determined times for assessment of the temporal progression of disease and lesions in the CNS were analyzed by light and electron microscopy.

MATERIALS AND METHODS

Mice

Female SJL/J mice, five to eight weeks of age, were purchased from Jackson Laboratory, Bar Harbor, ME, and were immunized between ten and 14 weeks of age. They were housed in groups of two to four mice per cage and provided food and water *ad libitum*. When severe clinical disease was present, affected mice were given daily nursing care consisting of hand feeding and hydration.

Peptide Synthesis

As described previously (22), peptides were synthesized manually using the simultaneous multiple peptide synthesis ("teabag") method of Houghten (30) on a p-methylbenzhydrylamine resin, which yields peptides having C-terminal amides. After cleavage from the resin, peptides were purified by reverse phase HPLC on a VYDAC-Flow C_{18} preparative column (The Sep/a/rations Group, Hesperia, CA). After purification, each peptide eluted as a single peak and the composition of each was confirmed by amino acid analysis.

Immunization

On Day 0, groups of mice were immunized subcutaneously in the abdominal flanks with 100 nmol (ten mice/group), 50 nmol, or 20 nmol (eight mice/group each) of mouse PLP residues 139–151 HCLGKWLGHPDKF (100 nmol = 154 μg) or mouse MBP residues 91–104 VTPRTPPPSQGKGR (100 nmol = 194 μg). Each immunization included 200 μg *Mycobacteria tuberculosis* H37RA (Difco Laboratories, Detroit, MI) in an emulsion of 200 μl of an equal volume of water and incomplete Freund's adjuvant (Difco). Each animal was also injected intravenously with 0.75 × 10^10 *Bordetella pertussis* bacilli (pertussis vaccine, lot no. WF262, Massachusetts Public Health Biologics Laboratories, Boston, MA) on Days 0 and 3.

Clinical Evaluation and Animal Killing

From Day 7 onward, the mice were examined daily and disease severity scores were assigned as follows: 0 = no disease, 1 = decreased tail tone or slightly clumsy gait, 2 = tail atony and/or moderately clumsy gait and/or poor righting ability, 3 = limb weakness, 4 = limb paralysis, 5 = moribund.

Two mice sensitized with 100 nmol of PLP peptide and two mice sensitized with 100 nmol of MBP peptide were killed on Day 7. Two mice from each antigen and dose group were killed on Days 10 and 14 (n = 12 per day). Two PLP peptide-sensitized mice were killed when they became moribund on Day 15, and two PLP peptide-sensitized mice were found dead in their cages on Days 14 and 15. The remaining mice were killed on Days 21 (n = 9) and 28 (n = 10). One mouse died after pertussis injection.

Before perfusion, mice were anesthetized intramuscularly with 1.6 μg ketamine and 0.16 μg xylazine. One mouse of each sensitization-matched pair was perfused through a 25 gauge heparinized needle in the left ventricle with 2.5% (Day 7 only) or 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After perfusion, the CNS tissues were immersed in the same fixative for 24 hours at 4°C. Representative samples of cerebrum, midbrain, brainstem, and three levels of spinal cord were stored in cacodylate buffer at 4°C, and the remaining CNS tissues were embedded in paraffin. The second mouse in each pair was killed by cervical dislocation. The CNS tissues were removed, fixed in 10% phosphate-buffered formalin and embedded in paraffin.

Histologic Evaluation

Paraffin-embedded sections of CNS tissues (1 block/mouse) were stained with Luxol fast blue-hematoxylin and eosin (LFB-H&E). Inflammatory foci, identified as perivascular clusters containing at least 20 mononuclear cells, were counted in leptomeninges, gray matter, white matter, and choroid plexus. Areas of loss of Luxol fast blue parenchymal staining indicative of demyelination were measured using a Bioquant System image analyzer (R & M Biometrics, Nashville, TN) attached to an IBM personal computer. Microscopic fields with white matter lesions were selected from coded slides. The images were projected from fields magnified to 1,117× on a monitor and areas of white matter staining loss were outlined on a digitizer pad. Mean area per field and total lesion areas were determined for each mouse. Clusters of inflammatory cells only within Virchow-Robin spaces and vessel lumens were excluded from the measurements.

One Micrometer Sections and Electron Microscopy

Spinal cord and brainstem samples were minced into one mm³ pieces, and treated with 1.3% OsO_4 in 0.13 M sym-collidine buffer, pH 7.4, dehydrated in graded ethanol solutions, and embedded in Epon. Sections, 1 μm thick, and thin sections were cut on an LKB Ultrotome (LKB, Bromma, Sweden). One μm sections of eight samples each from mice killed on Day 7, 150 samples from eight PLP peptide-sensitized mice, and 26 samples from one MBP peptide-sensitized mouse were stained with toluidine blue and examined by light microscopy. Samples with optimal tissue preservation and inflammatory lesions were selected for electron microscopy. Thin sections were mounted on nickel grids and stained with uranyl acetate and lead citrate. Grids from 23 Epon blocks were examined with a Philips 301 electron microscope.

RESULTS

Clinical

No clinical signs were observed in any mice from Days 1 to 9. Beginning on Day 9 PLP peptide-sensitized mice exhibited signs of EAE (Fig. 1). Only three of six PLP peptide-sensitized mice killed on Day 10 had shown signs of EAE. All other PLP peptide-sensitized mice exhibited onset of clinical disease by Day 16. The disease progressed rapidly in these animals and they usually became paralyzed or moribund over a period of two to four days. The maximal clinical score averaged 4.3. There was no detectable effect of initial antigen dose on the day of onset or the severity of clinical disease. Three mice sensitized with the lowest dose of the PLP peptide had onset of signs on Day 10. Progression of severe clinical disease required the killing of three PLP peptide-sensitized mice on Day 15. With the exception of one PLP
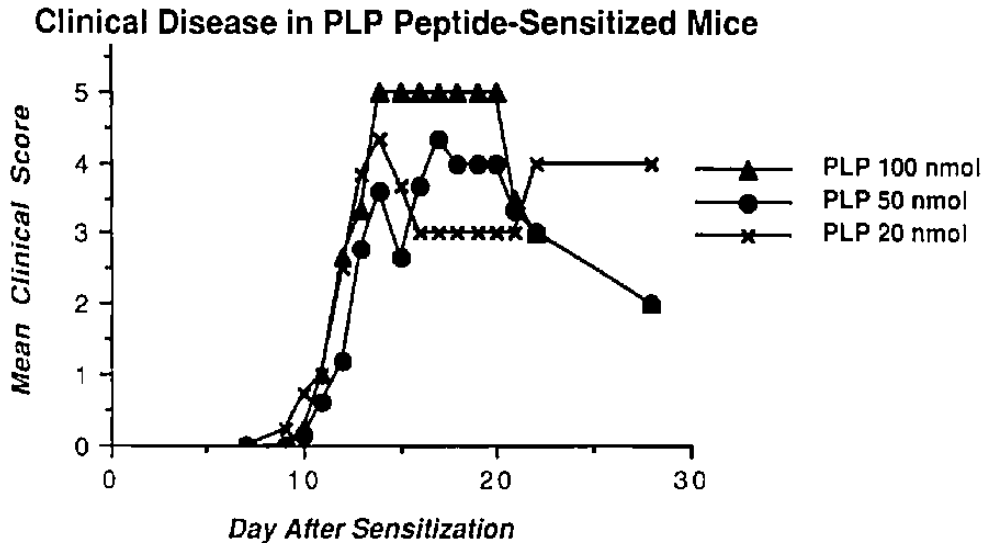
Clinical Disease in PLP Peptide-Sensitized Mice

Fig. 1. Mean clinical scores of groups of PLP peptide-sensitized mice. At the beginning of the experiment, there were eight to ten mice in each group. By Day 28, there were only one to two mice in each group.

peptide-sensitized mouse which was found dead in its cage on Day 14, CNS tissues of all mice were examined histologically.

By contrast, clinical disease was observed in only two mice sensitized with the MBP peptide and both had been given 100 nmol. These mice were well until Day 15 at which time they showed mild signs. In one mouse the disease progressed to a score of 4 and in the other to a score of 2. Both of these mice were killed on Day 21. No signs were observed in the other 22 mice sensitized with the MBP peptide.

Light Microscopy

In 22/22 of the PLP peptide-sensitized mice killed from Days 10 to 28, inflammatory infiltrates were present at all CNS anatomic levels. Lesions were most numerous in the spinal cord (Fig. 2A) and, in all mice, more foci were present in white matter than in gray matter. Most infiltrates were located in subpial areas and were contiguous with overlying leptomeningeal inflammation. There were minimal infiltrates in the choroid plexuses (0–3 foci). Lesions were frequently found in spinal cord nerve root entry zones, but did not extend into peripheral nerves (Fig. 2B) or cauda equina.

The infiltrates were comprised of mixtures of neutrophils, lymphocytes, and monocytes (Fig. 2C, D). In larger lesions, there were scattered macrophages containing phagocytosed myelin. Occasional swollen axons were also identified in spinal cord lesions. Fibrin deposition and red blood cell extravasation were not prominent features.

Quantitative data on meningeal and parenchymal inflammatory foci in PLP peptide-sensitized mice are shown in Figure 3. Mice sensitized with the PLP peptide and killed between Days 10 and 28 had means of 52 meningeal and 90 parenchymal inflammatory foci. Within the time period of the experiment, there was no apparent effect of initial dose of the PLP peptide or duration after sensitization on the numbers
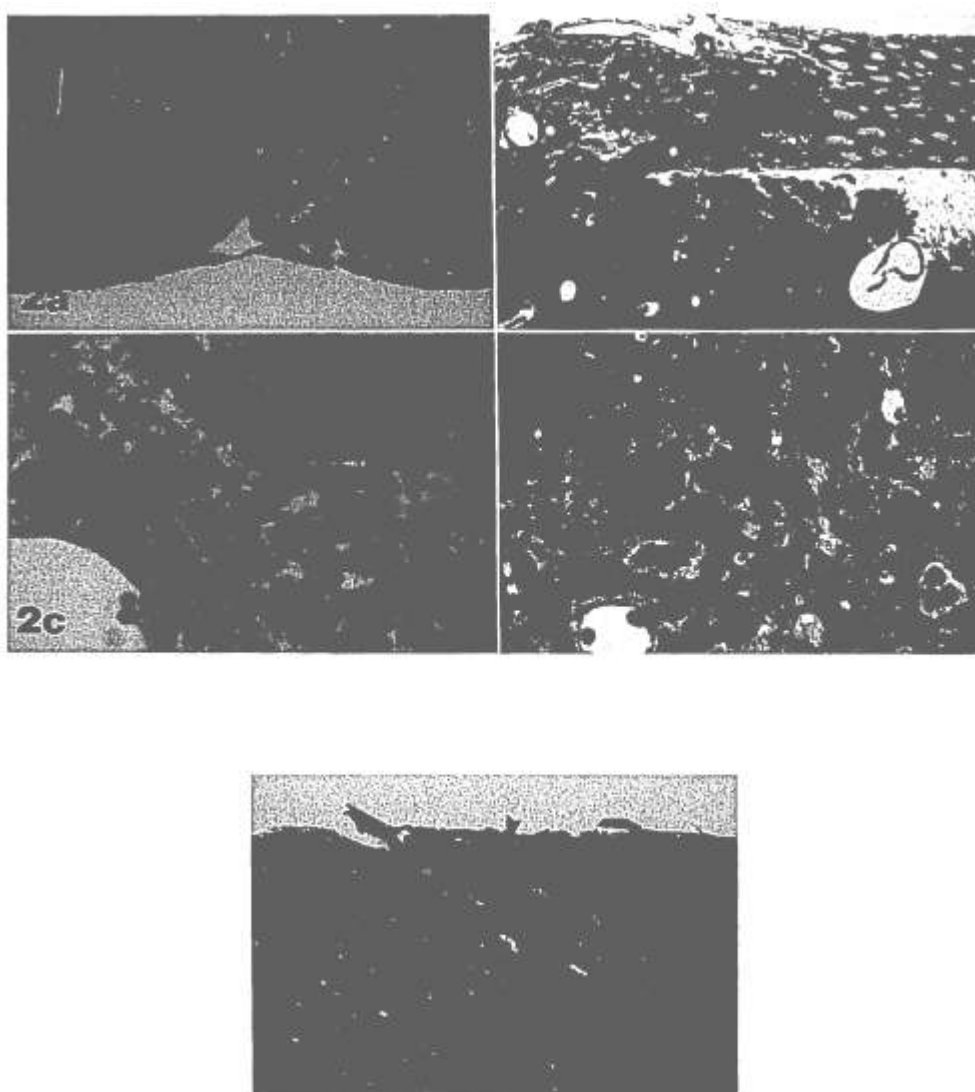
Fig. 2. Spinal cord lesions in PLP peptide-sensitized mice. A. Two foci of demyelination (arrowheads) in anterior spinal cord white matter in a mouse sensitized with 50 nmol of the PLP peptide and killed on Day 21. LFB-H&E. ×76. B. Posterior spinal cord of mouse sensitized with 100 nmol of the PLP peptide and killed on Day 14. There is inflammation in the spinal root entry zone (upper left), but not in the nerve root (upper right). 1 μm. Toluidine blue. ×764. C. Perivascular infiltrate in lesion of mouse sensitized with 20 nmol of the PLP peptide and killed on Day 10. Mononuclear cells and neutrophils (arrows) comprise the infiltrate. 1 μm. Toluidine blue. ×1,926. D. Lesion in mouse sensitized with 50 nmol of the PLP peptide and killed on Day 21. Mononuclear cells, edema, an axon without myelin (arrowhead), and myelin in vacuoles of a macrophage (arrow) are seen. 1 μm. Toluidine blue. ×1,926.

Fig. 4. Spinal cord of mouse sensitized with 100 nmol of the MBP peptide and killed on Day 21. Mononuclear cells predominate in the infiltrate and there is perivascular demyelination (arrowheads). LFB-H&E. ×123.

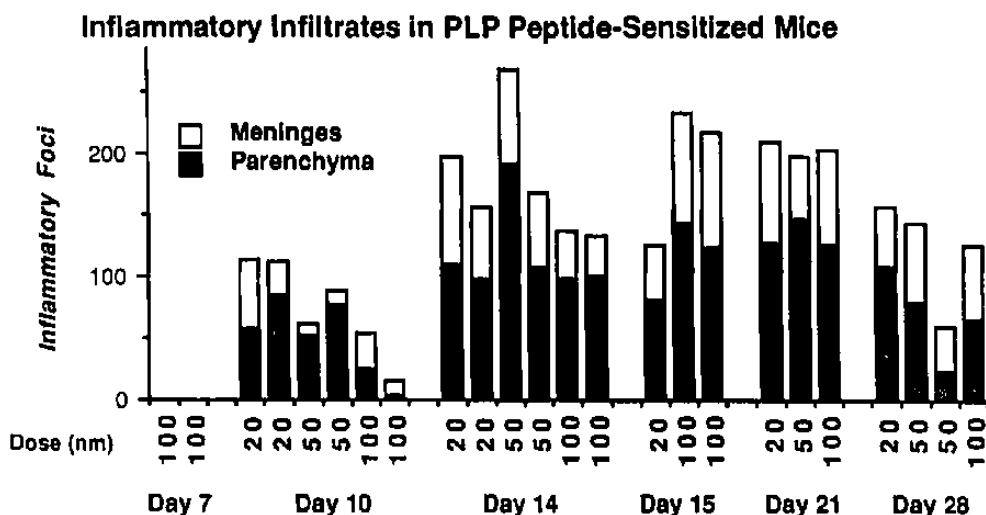## Inflammatory Infiltrates in PLP Peptide-Sensitized Mice

Fig. 3. Number of inflammatory foci in CNS parenchyma (gray matter + white matter, all anatomic levels) of PLP peptide-sensitized mice.

or appearance of inflammatory foci, i.e. foci in mice killed on Day 28 were indistinguishable from those in mice killed on Day 10.

Even though MBP peptide-sensitized mice showed minimal clinical disease, mild histologic EAE was observed in the majority of these mice. In MBP peptide-sensitized mice killed from Days 10 to 28, seven had only meningeal inflammatory foci (mean = 5 foci), one had four parenchymal foci, 12 had both meningeal and parenchymal foci (mean meningeal = 11 foci, mean parenchymal = 21 foci), and two had no CNS inflammation. Several mice had values comparable to those of PLP peptide-sensitized animals. The localization, cellular composition and histologic appearances of lesions in the MBP peptide-sensitized mice (Fig. 4) were the same as those in PLP peptide-sensitized mice.

Areas of parenchymal demyelination identified in paraffin-embedded sections correlated with clinical score at the time of death and with numbers of inflammatory foci in PLP peptide-sensitized mice (Fig. 5). There were no differences between glutaraldehyde-fixed and formalin-fixed tissues in amount of inflammation in paraffin-embedded sections. No abnormalities were found in the CNS of mice sensitized with either MBP or PLP peptides and killed on Day 7.

Electron Microscopy

Ultrastructural analysis of CNS lesions in PLP peptide-sensitized mice confirmed the presence of mixed mononuclear cell and neutrophil infiltration in the CNS (Fig. 6A). Within the lesions, there were numerous macrophages which contained abundant membranous material in vacuoles, consistent with phagocytosed myelin in lysosomes. These were frequently adjacent to axons which lacked myelin, suggesting that demyelination had occurred. The lesions also showed perivascular edema and prominence of glial fibers in astrocytes (Fig. 6B). Ultrastructural features of the parenchymal lesions in the single MBP peptide-sensitized mouse with severe clinical and histologic disease were essentially identical to those in PLP peptide-sensitized mice (Fig. 7).

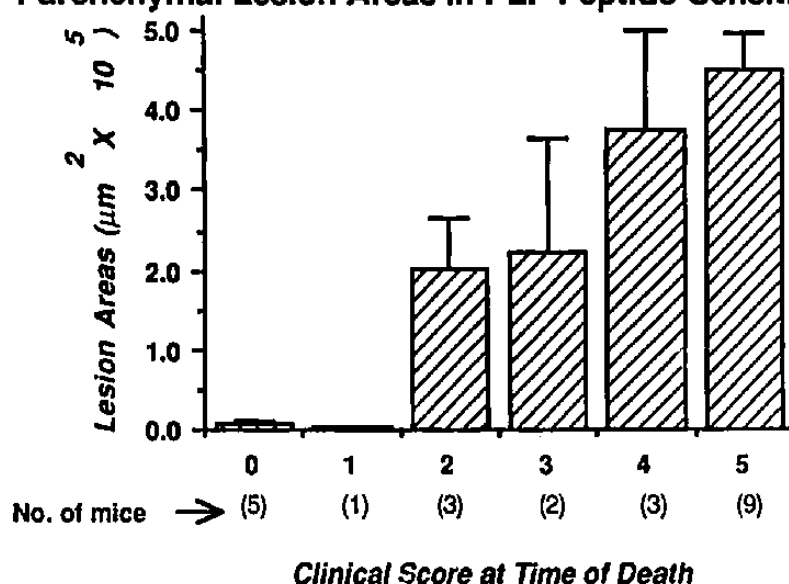## Parenchymal Lesion Areas in PLP Peptide-Sensitized Mice

Fig. 5. White matter lesion areas in paraffin sections determined with an image analyzer. PLP peptide-sensitized mice are grouped according to clinical score at the time of death. Bars indicate SEM.

DISCUSSION

This study documents that the immune response to as little as 20 nmol of PLP peptide 139–151 is sufficient to produce all of the morphologic as well as clinical features of acute EAE induced by whole spinal cord homogenate, whole PLP, whole MBP, and an MBP peptide in the majority of SJL/J mice. Clinical incidence, time of onset, temporal progression, and lesion severity, distribution and morphology were indistinguishable from acute EAE in spinal cord homogenate-sensitized (31) and whole bovine, guinea pig, rat, or mouse MBP-sensitized (23, 24, 29, 32) SJL/J mice. Acute EAE induced by sensitization with the PLP peptide was also clinically identical to that induced with whole bovine PLP (12), but the disease was pathologically more severe (mean inflammatory foci in mice given 13.3 nmol of whole PLP = 61; mean in mice given 20 nmol of the PLP peptide = 153). This difference is possibly attributable to the use of somewhat higher doses of antigen as well as *B. pertussis* (33) in the present study.

In contrast to PLP, in which only a single encephalitogen has been identifed to date in SJL/J mice, several epitopes of MBP are encephalitogenic in this strain. We selected mouse MBP peptide 91–104 for this study because it had been reported that sensitization with 20 nmol of this peptide induced acute clinical EAE in six of ten SJL/J mice (25) and it is of a size close to that of the 13 amino acid PLP peptide. In spite of the use of pertussis, only mice sensitized with 100 nmol (and not lower doses) of the MBP peptide exhibited clinical EAE and when signs were present, there was a later onset and they were less severe. Furthermore, severe histologic lesions were found in only two MBP peptide-sensitized mice. A longer, non-purified peptide preparation may have induced a higher disease incidence, but the shorter peptide minimized the induction of immune responses to multiple nested epitopes (34) and was an appropriate choice for comparison to the PLP peptide. Considerable vari-
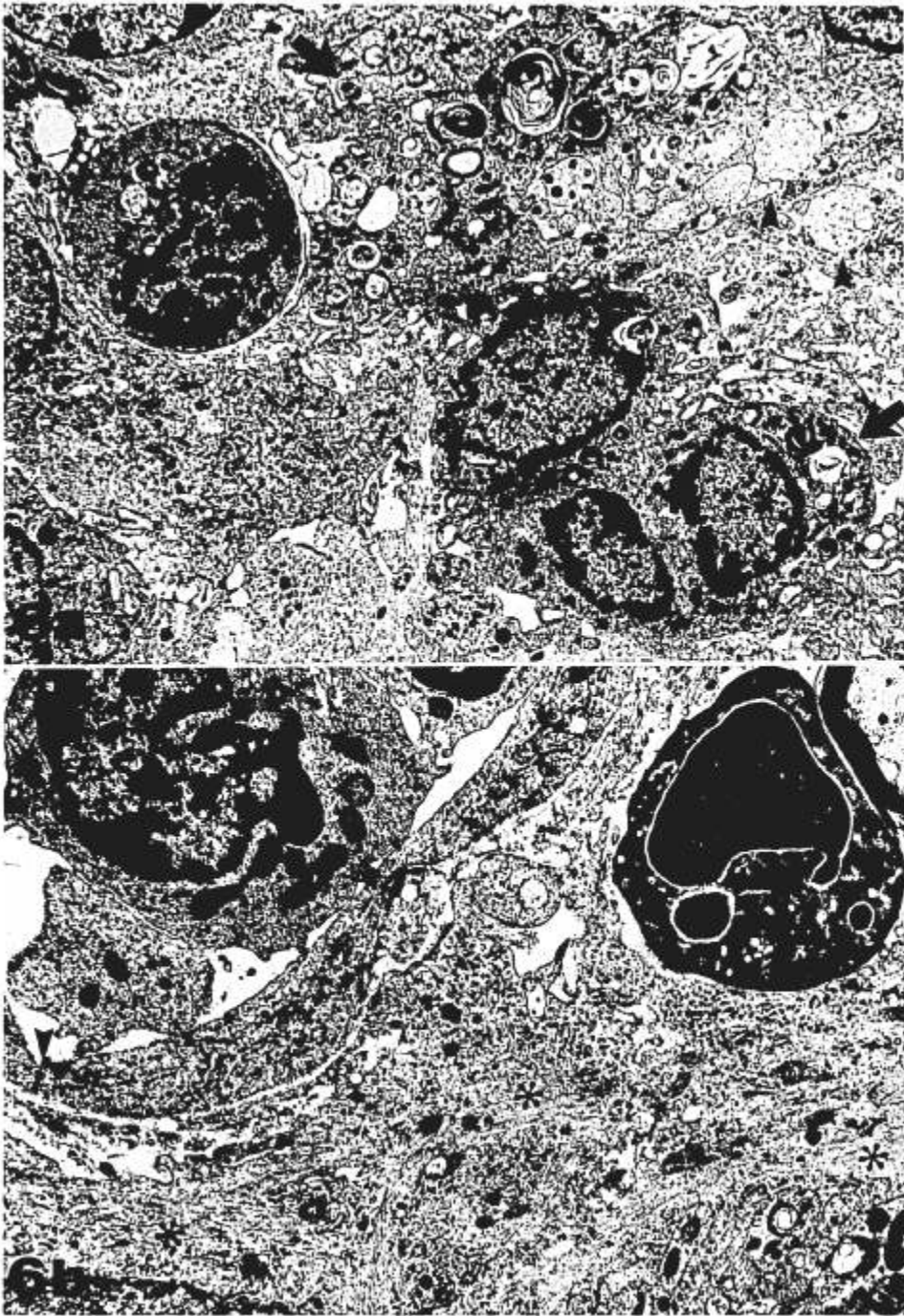
Fig. 6. Ultrastructure of lesions in PLP peptide-sensitized mice. A. Spinal cord lesion in mouse sensitized with 50 nmol of the PLP peptide and killed on Day 21. Macrophages with membranous inclusions suggesting ingested myelin (arrows) are adjacent to unmyelinated axons (arrowheads). ×5,500. B. Perivascular lesion adjacent to that in Figure 6A. A monocyte is present in the vessel lumen (upper left corner) and a polymorphonuclear leukocyte abuts a myelinated axon (upper right corner). Edema surrounds the vessel. An endothelial cell tight junction (arrowhead) and prominent glial filaments (*) are also seen. ×11,400.
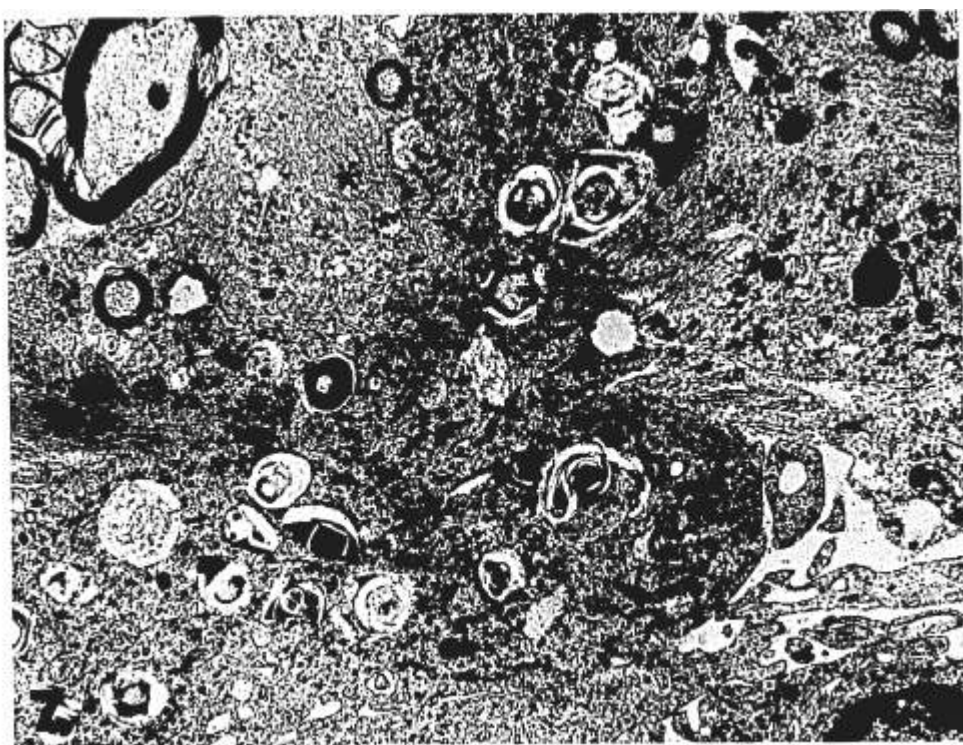
Fig. 7. Spinal cord of MBP peptide-sensitized mouse shown in Figure 4. Edema, membranous material in macrophage cytoplasmic vacuoles, and prominent glial filaments (*) are found in the lesion. ×12,780.

ability in the incidence and severity of EAE induced with single peptides of ten to 14 amino acids of MBP in SJL/J mice has also been found by others (25, 26). However, because histologic findings have not been analyzed in detail in any studies, it is not possible to compare our findings on the morphology of acute EAE induced with mouse MBP synthetic peptides with those of others.

As in other models, lesions in acute EAE in mice induced with the PLP peptide are predominantly found in CNS white matter, with relative sparing of both gray matter and the peripheral nervous system. The loss of myelin staining observed by light microscopy can be correlated with several ultrastructural features of the lesions. These include edema, decreased density of fibers by displacement by the cytoplasm of inflammatory cells, increased glial fiber prominence, and myelin phagocytosis. Although in whole MBP-sensitized SJL/J mice, the degree of demyelination has been related to the amount of antigen in the inoculum (29), we did not observe a similar dose dependency of the PLP peptide. It is likely that doses smaller than 20 nmol of the peptide would have resulted in less severe histologic disease. In this study, lesions in MBP peptide-induced EAE were much less common but, to the degree that we could analyze them, were also identical to those in other mouse acute EAE models, specifically with respect to composition of inflammatory infiltrates and extent of demyelination.

Demyelination is the feature of EAE which is central to its usefulness as a model of human multiple sclerosis (MS). Infiltration of MBP-specific T cells into the CNS is sufficient to induce demyelination in mice (20, 36), but additional myelin com-

ponents and antibody responses have been implicated in lesion size and extent of demyelination (35–39). However, myelin antigen-specific T cell responses may not be essential for primary demyelination (40). Furthermore lymphokines, such as tumor necrosis factor, the products of any activated T cells could also have direct pathologic effects on myelin (41).

The presence of potent encephalitogens from two different myelin proteins in one inbred mouse strain underscores the immunologic complexity of whole CNS tissue-induced and myelin-induced EAE models. Since immune responses to peptides of either MBP or PLP are characterized by common clinical and morphologic disease patterns in SJL/J mice, it is likely that epitopes of these CNS myelin proteins might induce comparable reactions in members of out-bred populations with different, and perhaps even more diverse susceptibilities. Pathogenetic mechanisms in MS are believed to be similar to those in EAE and in both diseases susceptibility is influenced by genetic factors. Thus, the genetically determined immunologic role of MBP in MS, under appropriate conditions, could also be played by PLP. However, because the properties of the PLP molecule are different from those of MBP, additional mechanisms might be important. The present observations have implications for the possible use of peptides as immunotherapeutic agents (42–47), and, in particular, point to the possibility that T cell responses to multiple myelin antigens may contribute to a chronic disease in genetically heterogeneous individuals, such as humans with MS.

ACKNOWLEDGMENTS

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