Spinal Cord Neuropathology in Rat Experimental Autoimmune Encephalomyelitis: Modulation by Oral Administration of Myelin Basic Protein

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Abstract. Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system (CNS) in which clinical neurological signs and histopathological changes of disease can be suppressed by feeding CNS myelin proteins. Using immunohistochemistry and image analysis, the cellular immune response was quantified over the rostral-caudal axis of the spinal cord in rats with EAE and in animals fed high- or low-dose myelin basic protein (MBP) prior to inducing EAE (tolerized animals). In a subset of rats, MBP was fed 9 days after MBP immunization to examine the effect of oral tolerance on the progression of CNS pathology. In unfed rats or rats fed vehicle only, activated microglia and macrophages were co-localized with T-lymphocytes throughout the spinal cord, but greater cellular reactions were evident in gray matter relative to white matter. In all tolerized animals, the CNS inflammatory response was reduced relative to controls. Subtle pathologic changes were occasionally observed in the CNS of MBP-fed animals, but the distribution of inflammatory cells in the rostral-caudal axis was more polarized in animals fed high-dose MBP. In this group, more T-cells and activated microglia were present in the dorsal spinal cord, specifically in the gray matter. In the group fed MBP after disease induction, clinical disease progressed as in control non-fed rats, but recovery from disease appeared to be accelerated. Thus, the results presented here provide a comprehensive analysis of the distribution and magnitude of inflammatory cells within the spinal cord in EAE and challenge the theory that MBP-induced EAE is only a white matter disease. These data also describe how the activation and distribution of immune effector cells is altered by oral tolerance and may help predict a range of neurological deficits not previously appreciated in EAE, particularly those effected by gray matter pathology.

Key Words: EAE; Image analysis; Macrophage; Microglia; Oral tolerance; T-lymphocyte.

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) serves as an animal model for the study of the human demyelinating disease multiple sclerosis (1, 2). As an autoimmune disease of the central nervous system (CNS), EAE is a useful paradigm for examining the sequelae of inflammatory cell entry and activation within the brain and spinal cord (3–5). Experimental autoimmune encephalomyelitis is known to be a T-lymphocyte-mediated disease in which myelin-reactive T-cells penetrate the blood-brain barrier (BBB) and orchestrate a local inflammatory reaction through the release of Th1 cytokines (e.g., IFN-γ, TNF-α, IL-2) (6–8). Subsequent tissue damage and demyelination are believed to be caused by the secretory products of activated macrophages and microglia (9, 10). Together, T-cells and macrophages/microglia represent the effector arm of the CNS cellular inflammatory response in EAE.

In studying therapeutic approaches in EAE, it is important to determine how various treatment modalities affect CNS immunopathology. Most studies use histological stains (e.g., hematoxylin-eosin) to assess the extent of inflammatory lesion distribution and then attempt to correlate relative lesion number with the effectiveness of treatment. While these techniques facilitate visualization of perivenular inflammatory clusters, it is difficult to determine how different subsets of effector cells are influenced by the treatment. In many instances, only parts of the CNS have been examined (e.g., only lumbar or sacral spinal cord or brain without spinal cord).

In instances where the entire CNS is sectioned, inflammatory lesions are often quantified without reference to neuroanatomical orientation. This is problematic with longitudinal spinal cord sections where dorsal/ventral orientation can be difficult to ascertain. If the presence of inflammatory lesions is related to overall clinical deficit, then it is logical to examine specific cellular changes within regions of the CNS which correlate with specific neurological functions. Therefore, a goal of the present study was to establish a standardized sampling technique to quantitate microglial/macrophage activation and lymphocyte infiltration throughout the rostral-caudal extent of the spinal cord within functionally distinct anatomical regions. In an effort to better define the effector potential of the cellular immune response, we focused on the magnitude and regional distribution of microglial activation in parallel with macrophage/T-cell infiltration. All indices of neuropathology were determined at peak clinical disease to facilitate direct comparison with parallel studies examining the effect of oral tolerance on CNS neuropathology.

Previous work has demonstrated a decrease in clinical disease and CNS inflammatory pathology in rats and mice fed myelin basic protein (MBP) prior to EAE induction (11–14). The effectiveness of oral tolerance has largely focused on clinical disease suppression and reduction of T-cell function in vitro. The present study...
examines how the oral administration of MBP influences the development and progression (delayed feeding) of inflammatory cell pathology within the spinal cord as well as the changes observed in peripheral lymph nodes. A previous report described cellular changes in parallel with cytokine expression within the brain and the effect of orally administered MBP on these changes (15). However, as the spinal cord is the primary target of neuropathology in rat EAE, we focused our studies on inflammatory reactions in that location. The present data demonstrate that both high and low doses of orally administered MBP are effective in suppressing the development of CNS histopathology. These data argue against a role for regulatory T-cells acting in the target organ, i.e. the spinal cord, under conditions of low-dose tolerance (15, 16). Also, the distribution of observable neuropathologic changes in both high- and low-dose groups may provide some insight into the regional specificity or susceptibility of the spinal cord to CNS inflammation in EAE.

MATERIALS AND METHODS

Animals

Twenty-eight female Lewis rats (9–11 weeks; 150–175 g) were randomly assigned to one of 5 different experimental groups (high-dose oral MBP, low-dose oral MBP, delayed high-dose oral MBP, vehicle-fed, or EAE control; see Table 1).

Induction of EAE and Oral Tolerance

All protocols for EAE and oral tolerance induction have been described previously (11). Briefly, for EAE induction, animals received intradermal injections of an emulsion containing guinea-pig MBP (25 μg/animal) and complete Freund’s adjuvant (CFA with 4 mg/ml Mycobacterium tuberculosis Jamaica strain) into the footpads. MBP was prepared from guinea pig spinal cords according to the method of Swanborg (17).

For induction of oral tolerance, animals were fed either high (HIGH) (20 mg total; 5 mg ×4 over 8 days; n = 7) or low (LOW) (5 mg total; 1 mg ×5 over 9 days; n = 7) doses of guinea-pig MBP dissolved in sterile phosphate-buffered (PBS) saline (11). Control rats were fed PBS only (VEH) or were not fed prior to MBP immunization (EAE only) (n = 5/group). Animals fed MBP or PBS were challenged 1–3 days after the final feeding. An additional group of animals (n = 4) received a high-dose regimen beginning 9 days after EAE induction (d-HIGH). Although our previous studies in male rats utilized MBP fed in bicarbonate buffer and soybean trypsin inhibitor, more recent comparative data indicates that induction of oral tolerance in female Lewis rats was easier to achieve than in males and did not require the addition of bicarbonate buffer or soybean trypsin inhibitor (15).

To confirm tolerance induction, clinical neurologic signs were assessed beginning 9 days after MBP-CFA challenge using a 5 point clinical scale (1 = flaccid tail, 2 = ataxic gait, 3 = severe hindlimb weakness, 4 = bilateral hindlimb paralysis, 5 = moribund). Half points were given for deficits that fell between two major categories in terms of severity. Animals were sacrificed 14–16 days after MBP injection.

Tissue Preparation

Animals were anesthetized with a mixture of ketamine HCl (80 mg/kg) and xylazine (50 mg/kg) and then perfused intracardially with 100 ml of cold PBS (0.1 M; pH 7.4) followed by 300 ml of paraformaldehyde (2% prepared in 0.1 M phosphate buffer). Spinal cords and popliteal lymph nodes were removed. During dissection of the spinal cords, individual spinal segments were marked using the dorsal roots as landmarks. This

Fig. 1. Image analysis sampling technique and quantitation. Digitized image of a lumbosacral spinal cord section divided into right and left dorsal/ventral quadrants. The inflammatory foci of largest magnitude within each of 10 sample regions are indicated numerically. A model of the circular sample tool is also indicated in the left dorsal horn of the gray matter (a). High-power field of OX42+ microglial cells enclosed within sample tool (b). Pseudo-color computerized identification of immunolabeled microglia (c). The proportional area (PA) = the area occupied by microglia (green) divided by the total area of the sample tool (see Materials and Methods).
facilitated later assessment of spinal level for tissue sectioning and quantitation (see below). Once removed, spinal cords and lymph nodes were postfixed for 30 minutes (min), then rinsed and stored in 0.2M phosphate buffer overnight. The next day tissues were cryoprotected in a solution of 30% sucrose. Spinal cords were sequentially blocked in the transverse plane into 10–12 separate segments while maintaining the rostral-caudal orientation, and then were frozen on dry ice. Five or six of these blocks, representing each major level of the spinal cord (upper/lower cervical, upper/lower thoracic, lumbosacral), were placed together in cryomolds, embedded in OCT compound, and frozen on dry ice. These preparations allowed simultaneous visualization of the rostral-caudal and dorso-ventral axis of the spinal cord on a single slide. Frozen molds were sectioned at 20 μ on a cryostat (Microm) and collected onto Superfrost slides (Fisher Scientific).

Morphological Techniques

Immunohistochemistry: The protocols used to stain rat CNS tissue are modified from indirect immunoperoxidase stains and have been described previously (18). Briefly, primary antisera applied to sections overnight at 4°C. On day 2, sections were rinsed (×2) with Tris-buffered saline (TBS; 0.5M, pH 7.6) and secondary mouse pre-absorbed biotinylated antisera (horse anti-mouse IgG, H+L chain; 1:400 dilution in PB; Vector Laboratories) applied overnight. The following day, tissues were rinsed with TBS (×2), and endogenous peroxidase activity was quenched using a methanol:hydrogen peroxide (30%) solution at a 4:1 ratio for 5 min. The ABC complex (Vector Laboratories) then was applied to each section for 1 hour at room temperature followed by TBS rinses (×3). Antibody binding was visualized using a commercially available DAB substrate solution (Vector Laboratories). Sections were dehydrated through ascending alcohols, cleared in Histoclear, and coveredlipped with Permount (Fisher Scientific). For selected sections, coverslips were removed in xylene and then rehydrated through descending alcohols into buffer. These sections were counterstained with cresyl violet acetate to facilitate visualization of nuclear morphology. Nonspecific or background staining was assessed by incubation of tissue sections in the absence of primary antibodies or in the presence of isotype-matched irrelevant primary antibodies (rat IgG; Sigma Immunohistochemicals; dilutions of 1:20–1:200).

Microglia, macrophages, and lymphocytes were visualized throughout the spinal cord using the following monoclonal antibodies (dilution; specificity): OX42 (1:500; complement type 3 receptor on microglia/macrophages), OX6 (1:500; monomorphic determinant of RT1B, MHC class II), and OX19 (1:200, CD5 molecule on T-lymphocytes). Other antibodies used but not quantitated include: OX39 (1:200; IL-2 receptor on activated T-cells) and ED1 (1:1000; cytoplasmic CD68-like protein associated with the lysosomal compartment in actively phagocytic macrophages).

Image analysis: For quantitative analysis of microglial activation and macrophage infiltration, computer-assisted standardized sampling techniques (MCID M4; Imaging Research Inc., Ontario, Canada) were developed to measure the area occupied by positively-labeled cells within a defined sample region, i.e. a proportional area (PA) measurement. A similar approach has recently been described for microglial/macrophage reactions after spinal cord injury (18). Briefly, to minimize variability within and between sections, two sections/animal spaced at least 200 μ apart were sampled for quantification within 10 anatomically defined spinal regions (right and left [R/L]) dorsal horn, R/L ventral horn, dorsal funiculus, ventral funiculus, and R/L lateral funiculi dorsal and ventral to the central canal). This procedure was repeated across all major spinal levels (e.g. cervical [C1-C6/C7-C8], thoracic [T1-T12], lumbosacral enlargement).

For sampling, digitized images of immunolabeled sections were contrast enhanced to clearly differentiate positive staining from background, and a range of optical densities was assigned that corresponded with positively stained tissue (Fig. 1). Within each region, a circular standardized sampling tool (100 × 100

Fig. 3. Microglial and macrophage distribution (OX42 immunoreactivity) in the spinal cord of rats with EAE (a–e) or rats fed high-dose MBP prior to inducing disease (f–j). Photomicrographs, a–e and f–j represent a progression from upper cervical spinal cord into lumbosacral levels. Note the presence of OX42+ expression throughout the gray matter (arrowhead) at all spinal levels in uninfected animals. Activated and reactive microglia were routinely observed around ventral horn motor neurons in the cervical enlargement (open arrow in a; C3) and at lumbosacral levels (open arrow in e, arrows). Activated microglia were present around neurons of the intermediolateral cell column (open arrow in C7/T13) and near the meninges or spinal veins in white matter (double arrowheads in a, d; d = T10–12). These latter cellular reactions were less prominent than those in gray matter. In tolerized animals, occasional activated microglia were localized to the gray matter and the roots of the dorsal spinal cord (pictured in j for the lumbar spinal cord, arrow). However, most microglia had ramified morphology. Scale bar = 480 μ.
mm²) was placed over the center of the largest inflammatory lesion. Within this sample region, the PA of stained tissue was measured.

The PA measurements are quantitative measures of changes in the magnitude of the cellular infiltrate and/or the activation state of the specified cells relative to the sample area. In the case of microglia/macrophages, both cellular hypertrophy and/or hyperplasia could result in increased PA measures. Since microglial hypertrophy signifies an increased state of activation, larger PA measurements indicate a cellular inflammatory response of larger magnitude. To facilitate comparisons between animals and between different levels of the spinal cord, PA measurements in experimental animals were normalized to PA measurements in identically stained spinal sections from control animals. Thus, reported PA measurements are expressed as activation indices (PA of experimental tissue/PA of control tissue).

Parenchymal and perivascular lymphocytic infiltrates were quantitated using areal density measures (18, 19). Using a Zeiss Axioplan microscope fitted with a monocular counting grid, T-cells were counted at high power (400×) in each of the 10 regions described above. An attempt was made to eliminate biased estimates by using serial optical sectioning techniques (19).

Classification of microglial morphology: The nomenclature used to describe microglial phenotype(s) at various stages of activation has been reviewed extensively and will not be reiterated here (20, 21). For the purposes of this report, ramified or resting microglia are elaborately processes cells (OX42+ and ED1/OX6-). Activated or reactive microglia (strongly OX42+, ED1+, and OX6+) are stout in configuration, with some remnants of cellular processes still present. These latter cells are morphologically distinct from the round ED1+ macrophages that contain small lipid inclusions, and are typically seen in or adjacent to blood vessels.

Statistics: A two-way analysis of variance (2-way ANOVA) was used to detect differences between clinical signs and treatment groups as a function of time after MBP challenge. Significant interactions between these variables were assessed using one-way ANOVA followed by Tukey’s multiple comparison test each day after challenge. Identical one-way ANOVA’s were performed for activation indices between spinal levels. Differences in T-cell number between gray and white matter were compared using Student’s two-tailed t-test. For all analyses, significance was set at p < 0.05.

RESULTS

Clinical Signs of Disease

Beginning 9 days after MBP-CFA injection (EAE induction), all animals were monitored for the development of neurologic signs (Fig. 2). All groups, with the exception of the high-dose MBP-fed rats, presented with clinical manifestations of disease by 11 days post-challenge. All animals in the EAE group developed full hindlimb paralysis over the course of 3–4 days. Similarly, all rats that were fed PBS (vehicle controls) developed clinical signs. In groups that were fed MBP prior to challenge, few animals developed clinical disease. In the high-dose group, 2/7 showed mild clinical signs compared with 4/7 showing clinical disease in the low-dose group. However, mean clinical scores were not statistically different between the 2 groups at any time. Feeding high-dose MBP beginning 9 days after MBP-CFA injection did not alter disease onset (mean maximum clinical score and number of animals with disease was identical to control groups), yet recovery from disease was accelerated in this group compared with EAE and VEH control groups (Table 1).

Neuroimmunopathology

The distribution and magnitude of cellular immunopathology varied between gray and white matter, dorsal and ventral spinal cord, and between spinal levels. In apparent contradiction to the notion that inflammatory reactions predominate within white matter tracts during rat EAE induced by MBP/CFA sensitization, the present data revealed a larger microglial/macrophage reaction and T-cell infiltrate within gray matter at all spinal levels examined (Figs. 3, 7, 8). Activated microglia, T-cells, and macrophages surrounded interneurons and motor neurons in the intermediate gray matter and ventral horns at all levels. Inflammatory lesions also were consistent in the intermediolateral cell column at thoracic spinal levels (Fig. 3c). In tolerized animals showing inflammatory changes in the CNS without overt clinical disease, activated microglia and lymphocytic infiltrates were predominantly localized to the subpial parenchyma of the dorsal horns and less frequently to the subpial white matter. This polarization of inflammatory reactions in the dorsal spinal cord was most profound in the high-dose MBP-fed group (Figs. 5, 7, 8).

Microglia and macrophages in EAE/VEH control groups: In all animals showing clinical signs, expression of OX42 immunoreactivity was increased throughout the rostral-caudal spinal axis (Fig. 3). Activated and reactive microglia were interspersed with phagocytic macrophages (as indicated by ED1 immunoreactivity) and frequently were distributed around blood vessels, spinal

Fig. 4. Comparative microglial/macrophage morphology in rats with EAE or tolerized rats. All tissue sections, with the exception of section (e), were stained with OX42. (a–f) In animals with EAE only, activated microglia (a, c, d–f) and macrophage clusters (arrow in b) are found throughout gray (a–e) and white matter (f). Note the gradient of microglial activation distal to the center of the mononuclear lesions in (open arrow in a, d, and e) and (d, e). It is apparent that only a subset of microglia/macrophages upregulate MHC class II molecules (compare OX42 and OX6 labeling in d and e, respectively; adjacent tissue sections). In tolerized animals (g–j), ramified/resting microglia are found within gray (g–i) and white matter (j). Compare morphology of microglia in a/g, c/h–i, and f/j). Scale bar = 90 μ (a); 460 μ (b, c, f, g, i, j); 180 μ (d, e, h).
nerve roots, and the dorsal and ventral root attachments (Figs. 3–5). Prominent microglial/macrophage clusters surrounded endothelial profiles in the ventral horns of the gray matter and the intramedullary veins draining the gray matter. Apparent gradients of microglial ramification occurred in the parenchyma at a distance from lesion foci (Fig. 4a).

The distribution of MHC class II-positive cells was nearly identical to that seen with the OX42 antibody, indicating that microglia/macrophages are the predominant cell types expressing MHC class II molecules. However, we cannot exclude the possibility that some MHC class II-immunoreactivity was present on reactive astrocytes and endothelia. Visible gradations in staining patterns, indicative of morphological activation and upregulated MHC class II expression, were more obvious than with OX42 (Fig. 4d, e). The lumbosacral spinal cord showed the largest increase in MHC class II immunoreactivity despite prominent microglial activation at all spinal levels (increased ED1 and OX42 concomitant with morphological transformation) (Figs. 5, 6).

Microglia and macrophages in oral tolerance groups: Most OX42+ cells in the parenchyma were ramified microglia (Fig. 4g–j). However, as indicated above, some tolerant animals showed traces of neuropathology, i.e. activated and/or reactive microglia were found in the spinal cord of animals fed high- and low-dose MBP without apparent clinical neurologic signs. When present, cellular changes were mostly in the dorsal horns and consisted of activated microglia colocalized with T-lymphocytes. Phagocytic macrophages (ED1+ cells) were rarely seen in these animals.

Expression of MHC class II molecules was absent or significantly reduced throughout the spinal cord of animals fed MBP (Figs. 5, 6). In animals showing minimal pathology, MHC class II expression was co-localized to regions of microglial activation and increased T-cell infiltration. Again, these cellular reactions were usually in the dorsal spinal cord. In naive and tolerant animals without apparent histopathologic changes, constitutive MHC class II immunoreactivity was restricted to the meninges and parenchymal perivascular cells/microglia (Fig. 5a, b).

Lymphocytes in EAE/VEH control groups: T-cells (OX19+) were scattered throughout the spinal cord of both EAE and vehicle-fed groups, with the largest number of T-cell clusters found around penetrating branches or collaterals of the ventral and dorsal median spinal veins as well as around the venous plexi of the pia mater. Similar to observations of microglial/macrophage activation, there were more T-cells within the gray matter at all spinal levels, although lumbosacral levels contained larger numbers of total T-cells (Figs. 7, 8). Adjacent sections confirmed elevated IL-2 receptor expression (OX39+) on a subset of these cells (Fig. 7b). Sections adjacent to those used to identify T-cell infiltrates always corresponded with regions of activated microglia and macrophages (morphologic changes and increased MHC class II expression).

Lymphocytes in oral tolerance groups: In tolerized animals, T-cell numbers were reduced at all levels of the spinal cord. Unlike EAE and vehicle-fed control groups, in high/low dose fed animals, T-cells were more evenly distributed between gray and white matter (Fig. 8b). However, T-lymphocytes were not evenly distributed in the dorso-ventral axis of animals fed high-dose MBP (Fig. 8c). Similar observations of a polarized dorsal/ventral T-cell distribution were seen in the low-dose group, but with less frequency. Again, similar to observations of microglial activation, in tolerized animals these T-cell clusters tended to be associated with small diameter vessels in the superficial dorsal horn or as cellular aggregates associated with the pial surface.

Both high- and low-dose oral MBP affected the number and activation state of T-cells in the lymph nodes, draining the site of immunization (i.e. popliteal lymph nodes) (Fig. 9). In the lymph nodes of EAE and vehicle-fed control animals, T-cells were densely packed, making it difficult to distinguish individual cells. In both oral tolerance groups, T-cell density was decreased. However, as indicated by the presence of OX6+ cells with blast-like morphology, activated T-cells were reduced in number in MBP-fed rats relative to controls.

Effects of delayed feeding: All animals receiving delayed high-dose MBP treatment showed accelerated recovery from clinical disease (Table 1). In each case, evidence of inflammatory reactions was still present. Microglia were morphologically distinct from the activated or reactive microglia seen in EAE and VEH control groups and reduced upregulation of MHC class II molecules were noted in the CNS of fed rats (Fig. 10). However, the total number of T-cells in the CNS of this group was similar to that of control groups (Fig. 8c).

DISCUSSION

Historically, quantitation of histopathologic changes and correlation with clinical disease has proven difficult because of the random localization of inflammatory lesions in the EAE spinal cord. Results presented here indicate that by using comprehensive sampling techniques, quantitative variability can be reduced and inflammatory reactions can be measured and compared between gray/white matter and dorsal/ventral spinal cord within a specified spinal level. The data indicate that the magnitude and distribution of inflammation are dependent upon the spinal level and section orientation. Other laboratories have shown that the lumbar spinal cord is a primary site of T-cell infiltration in rats with acute EAE (3, 4). However, these studies did not maintain a quantitative delineation of the cellular infiltrates within specific anatomical
Fig. 5. MHC class II expression (OX6) in rats tolerized by the oral administration of MBP prior to challenge with EAE (a, b, c) or EAE challenged only (c, d). Note the absence of MHC class II immunoreactivity in the lumbosacral spinal cord of animals fed high-dose MBP prior to inducing EAE (a; arrow indicates ventromedian fissure). In most tolerized animals, only cells within the spinal roots or within the meninges stain positive (b; r = dorsal root; arrowheads delineate meninges). Parenchymal staining is absent (b). In EAE animals, MHC class II staining was distributed throughout the spinal cord in a pattern similar to the OX42 staining (c, d; also see Fig. 3). Some tolerized animals exhibited histopathology, usually localized in the dorsal horns (e; high-dose animal). Scale bar = 360 μ (a); 25 μ (b); 400 μ (c); 45 μ (d); 500 μ (e).
regions of the spinal cord. Our data show that microglial/macrophage activation and T-cell influx are of larger magnitude in the spinal gray matter compared with white matter, especially around neurons in the ventral horn and within the superficial lamina of the dorsal horns. In rats fed MBP prior to EAE induction, activation of microglia (assessed by morphology and expression of MHC class II) was suppressed and T-lymphocyte/macrophages, when present, were more evenly distributed between gray and white matter within a spinal level. In these tolerized animals, inflammatory changes were localized to the dorsal half of the spinal cord, usually within gray matter of the dorsal horns. The tendency for inflammatory changes to occur in the dorsal spinal cord was most pronounced in animals fed high-dose MBP prior to inducing EAE. This group of animals showed fewer motor deficits compared with other groups. From these data, it appears that the distribution of T-cells and activated microglia/macrophages may help predict changes in neurological function.

Myelin proteins are present in spinal cord gray matter (22–24), and thus CNS-reactive T-cells could infiltrate this region as a result of target antigen recognition. Indeed, some inflammatory foci are located within regions where MBP immunoreactive fibers penetrate the gray matter (not shown). However, these macrophage/lymphocyte clusters are more prevalent along blood vessels and nearby neurons and are not always found adjacent to or surrounding myelinated fibers. Thus, the predilection for inflammatory cell influx into the gray matter in rat EAE may also be influenced by physiological variables (e.g., distribution and density of the microvasculature). There is an uneven distribution of blood vessels throughout the spinal cord, with more vessels supplying cervical and lumbar spinal levels (25, 26). Within a spinal level, the highly metabolic gray matter receives a more generous blood supply than white matter and nearly 3 times as many vessels supply the dorsal half of the spinal cord compared with the ventral half (25, 26). These differences are magnified across spinal levels as the proportion of gray to white matter changes, i.e., more gray matter is found in the cervical and lumbar spinal cord relative to thoracic levels. Since leukocytes enter the CNS primarily via the vasculature, it is not surprising that in all rats with EAE, inflammatory pathology was comparable within the cervical and lumbar spinal cord, while thoracic levels were less affected. Still, pronounced microglial activation and T-cell infiltration were present at thoracic levels, often around small neurons in the intermediolateral cell column. Why there were quantitatively fewer inflammatory changes at cervical levels in animals fed prior to disease induction (vehicle and MBP) is unclear, since the pattern and magnitude of T-cell influx in the delayed high-dose and EAE groups were nearly identical. It will be interesting to see if the stress/glucocorticoid response, which is likely to be elicited by anesthesia and feeding, alters inflammatory cell trafficking, adhesion molecule expression, and/or chemokine expression within the spinal cord.

From the present data, it is puzzling why gray matter inflammation and the potential consequence(s) of this pathology remain largely unexplored in EAE. As alluded to
Oligodendrocyte glycoprotein–specific T-cells is distinct from that of MBP-induced EAE (27). In myelin oligodendrocyte glycoprotein–induced disease, a decrease in the macrophage:T-cell ratio was reported in the CNS, and these animals did not develop clinical disease. A recent study has suggested that the antigen specificity of T-lymphocytes dictates the regional distribution of inflammatory lesions within the CNS (28). Thus, it is possible that the predominant grey matter inflammation observed in this study is a consequence of the trafficking patterns and immune effector functions specific to immunization with guinea pig MBP. However, this does not explain the reports of axonal attrition and depletion of neuronal metabolites/neurotransmitters in both EAE and in multiple sclerosis patients (29–32). Perhaps cellular inflammatory changes in grey matter occur secondary to white matter damage (33, 34). In the present report, rapid activation of microglia and T-cell influx in the spinal cord grey matter could represent cellular reactions to antigen-specific inflammatory insults originating in white matter tracts located anterograde or retrograde to the neuronal cell bodies. Nevertheless, insults to normally functioning neurons and their axons, whether they are primary or secondary to white matter inflammation, could contribute to neurologic deficits. Indeed, in vivo magnetic resonance spectroscopic imaging indicates that the strongest pathologic correlation to clinical disease in multiple sclerosis is related to a decrease in neuronal metabolism (35, 36). Neuron and/or axon pathology may also serve to focus immune responses to regions of increased MHC class II immunoreactivity. This relationship has been shown in EAE, where inflammatory lesions were focused to sites of increased microglial MHC class II expression, specifically in Wallerian degenerating fiber tracts and near axotomized motor neurons (37, 38). How these neuropathologic changes are affected by immunomodulatory treatments has not been well characterized for spinal cord. Thus, in the present study we have examined how immunopathologic changes in the EAE spinal cord are affected by feeding high or low-doses of MBP.

Although different mechanisms have been proposed to explain high and low-dose oral tolerance, in our hands a reduction or elimination of CNS histopathology was equally impressive in animals fed either dose of MBP. While T-cell function was not directly measured, the reduction in cells bearing activation markers (IL-2 receptors and MHC class II markers) in the CNS and in peripheral lymph nodes suggests that both feeding regimens decreased the myelin-reactive T-cell repertoire below a level capable of producing significant CNS pathology. This observation appears to contradict the hypothesis of Khoury et al (1992) that suggested that amelioration of disease in rats fed low-dose MBP results from increased trafficking of regulatory T-lymphocytes with subsequent
release of immunosuppressive cytokines (e.g. TGF-β, IL-4) within the brain/spinal cord (15). While we did not characterize cytokine patterns in the CNS, we did not observe a correlation between the presence of T-cell infiltrates and protection from disease. Instead, the absence of clinical signs paralleled a decrease in T-cell number and microglial/macrophage activation, most notably a decrease in MHC class II expression. In the delayed high-dose group, attenuation of the microglial reaction without a parallel decrease in T-cell number may suggest a change in the cytokine profile of infiltrating cells—one that is more conducive to recovery from disease (i.e. a shift towards Th2-lymphocytes within the CNS).

Our data indicate that the differences in clinical disease observed between high- and low-dose–fed animals may result from the differential distribution of T-cells in the dorsal and ventral spinal cord (see Figs. 7 and 8). In animals fed high-dose MBP, fewer T-cell infiltrates were present within the ventral spinal cord compared with animals fed low-dose MBP. As myelin-reactive T-cells can

Fig. 9. Effects of oral tolerance on T-lymphocyte density (OX19; a–c) and activation in the popliteal lymph nodes. Compared with tissues from vehicle fed rats (a), feeding high- (b) or low-dose (c) MBP decreased lymphocyte density in lymph nodes. Micrographs represent lymphocytes within the lymph node germinal center. A general reduction in lymphocyte activation (T- and B-cell) is apparent in the lymph nodes of vehicle-fed (d) and high-dose, MBP-fed (e) animals (decreased MHC class II expression, OX6). Scale bar = 30 μ (a–c); 20 μ (d, e).
affect axonal conduction (39), the absence of T-cells around motor neuron pools may explain functional differences between the two groups. Future studies will determine if this difference in cellular distribution between the high- and low-dose groups is related to altered expression of adhesion molecules and chemokines throughout the CNS neouraxis.

When present in tolerized animals, activated microglia and T-lymphocytes were seen within the superficial lamina of the dorsal horns. Less frequently, small submeningeal lesions were observed in the adjacent white matter of the dorsal spinal cord. Why inflammatory cells predominate in these regions is uncertain. However, it is interesting that this is a watershed area for capillary beds converging from the dorsal and ventral spinal cord and thus may be a region vulnerable to histopathologic insult (26, 27). Since these changes occur in a region of the spinal cord associated with pain/ailodynia and hyperexcitability, behavioral measures to assess these neurological symptoms may provide a useful adjunct to measures of motor function. Such techniques may be especially valuable in determining the relative integrity of neural function in EAE when there are no indications of paralysis.

Existing literature and the present data suggest that a crude ordinal scale to measure EAE may not correlate with neuropathological changes and therefore may underestimate the extent of disease. Furthermore, there is recovery of motor function in the face of persistent neuropathology. The presence of activated microglia and T-cells around neurons in the intermediolateral cell column (see Fig. 3C) question the integrity of autonomic function in animals with EAE. To our knowledge, autonomic dysfunction has not been tested in EAE. However, abnormal autonomic reflexes have been described in multiple sclerosis patients (40). Thus, a combination of more sensitive behavioral paradigms may define the progression of neurological dysfunction in EAE more accurately than one which measures only motor systems.

As the effector functions of microglia, macrophages, and T-cells are likely to affect the survival and function of neurons and glia, the present study should facilitate future correlations between behavioral outcome measures and CNS neuropathology. Subtle differences in regional CNS pathology in animals orally tolerized with high or low-dose MBP provide a basis for studying the effects of oral tolerance on the efferent arm of immunological activation within the CNS. Specifically, it will be interesting to determine if signaling molecules are differentially regulated between the two paradigms. Based on proposed mechanistic differences between oral and parenteral tolerance induction in EAE (41), it also will be important to determine to what degree neuropathologic changes are affected by these different protocols and how the resultant neuropathology correlates with measures of behavioral outcome. In this way, we may begin to understand the relationship between immunological activation and onset of neurologic disease.

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Fig. 10. Effects of delayed high-dose oral tolerance on microglial activation (OX6). Relative to EAE spinal cord (a, c), note the reduced expression of MHC class II molecules when MBP is fed 9 days after inducing EAE (b, d). High-power photomicrographs demonstrating relative level of microglial activation in EAE control (c) and d-HIGH group (d). Micrographs in (c) and (d), taken at the gray/white matter junction of the dorsal horns (delineated by arrowheads), show the relative decrease of MHC II and a less reactive morphology of microglia in the d-HIGH tissue. Scale bar = 100 μm (a, b); 50 μm (c, d).


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