Quantification of Myelin and Axon Pathology During Relapsing Progressive Experimental Autoimmune Encephalomyelitis in the Biozzi ABH Mouse

Samuel J. Jackson, PhD, JangEun Lee, PhD, Maria Nikodemova, PhD, Zsuzsanna Fabry, PhD, and Ian D. Duncan, BVMS, PhD

Abstract

Multiple sclerosis is an immune-mediated demyelinating disease, with axonal loss underlying long-term progressive disability. In this study, we have analyzed axonal and myelin pathology in a chronic relapsing-remitting experimental autoimmune encephalomyelitis model in Biozzi ABH mice induced by immunization with a syngeneic spinal cord homogenerate. The animals were followed for 3 months; inflammation, T-cell infiltration, demyelination, and axonal loss were examined at various time points throughout the disease course. We found that macrophage infiltration and microglia activation preceded detectable T-cell infiltration. Axonal loss was first evident at the acute phase of disease before demyelination was detected. Demyelination and axonal loss occurred after each relapse and correlated with increasing residual motor deficits in remission. The resulting lesions displayed evidence of demyelination, remyelination, axonal degeneration, and axon loss. After a series of 3 relapses, animals entered a chronic progressive phase with permanent paralysis and a relative absence of inflammation. Axonal loss continued in this phase, although demyelinated axons persisted. These findings indicate that experimental autoimmune encephalomyelitis in Biozzi ABH mice has important similarities to multiple sclerosis with a relapsing-remitting disease course followed by a secondary progressive phase; it is thus a suitable model in which to explore remyelination and neuroprotective therapies for multiple sclerosis.

Key Words: Axonal loss, Chronic relapsing, Demyelination, Experimental autoimmune encephalomyelitis (EAE), Inflammation, Multiple sclerosis, Neurodegeneration.

INTRODUCTION

Multiple sclerosis (MS) has been historically considered as an immune-mediated, demyelinating disease. Today, however, it is well established that axonal loss, likely triggered by the initial immune attack, is responsible for long-term disability in MS patients (1, 2). Although there are several clinical forms of MS, more than 80% of patients develop relapsing-remitting disease usually followed by a chronic-progressive phase (3). Current therapies of MS are predominantly focused on the inhibition or modulation of the immune responses, thus far with limited effectiveness. Thus, it has become evident that successful treatment must address both components of the pathology—neuroinflammation and neurodegeneration (4). Investigation of such treatment strategies requires animal models that closely reproduce the complexity and heterogeneity of MS pathology (5). Experimental autoimmune encephalomyelitis (EAE) has been used extensively as a model of MS; it displays many pathological similarities to the human disease, including infiltration of lymphocytes into the central nervous system (CNS) parenchyma, inflammation, and demyelination (6). A recent study of EAE in Dark Agouti rats described axonal loss during the course of disease that highly correlated with clinical severity in this model (7). In other EAE models, such as in the myelin oligodendrocyte glycoprotein (MOG) peptide 35–55–induced model in C57BL/6 mice, early axon loss was detected in parallel with immune cell infiltration before visible changes in the behavioral signs of EAE (8). These studies emphasize that establishing suitable animal models of CNS autoimmune disease in which axonal and myelin integrity can be defined is critical for the development of neuroprotective strategies for humans.

The value of EAE in MS research has been debated because of differences between the disorders and, notably, because of a failure to translate to MS some immunomodulatory therapies that ameliorate EAE (9). Three current therapies that benefit MS (i.e. glatiramer acetate, mitoxantrone, and natalizumab) were, however, first tested in EAE. If used judiciously, EAE remains the most useful model for developing a better understanding of the pathophysiology and testing potential therapy of MS (10). Many of the therapies that are effective for counteracting inflammation and immune-mediated demyelination in early EAE do not affect progressive MS, which is refractory to immunotherapy (11, 12). There is increasing evidence that axon loss occurring at the chronic-progressive phase of MS is independent of neuroinflammation and that neuroprotective rather than anti-inflammatory treatment strategies are needed (13, 14).
Many EAE models are induced by immunization with different neuroantigens, most commonly myelin proteins or peptides. Myelin oligodendrocyte glycoprotein peptide residues 35-55 induce chronic disease after a single attack in C57BL/6 and Biozzi ABH mice (15). In the Dark Agouti rat, immunization with MOG 1–125 induces a biphasic disease (16). In contrast, after immunization with mouse spinal cord homogenate, Biozzi ABH mice develop a relapsing-remitting disease followed by slow worsening of signs (6, 17, 18); this progression is not dependent on CNS autoimmunity, as appears to be the case in MS. The disease that develops during a period of weeks to months is associated with significant axonal damage (6, 18); it may, therefore, provide a sufficiently wide window to test therapies that might ameliorate disease progression. A good model of relapsing-remitting disease with apparent early- and late-stage axonal loss will be valuable for testing therapies aimed at preventing the late stage of disease. This requires a detailed knowledge of the temporal sequence of demyelination and axon loss in the model along with the role that inflammation plays at different stages of the disease.

In this study, we characterized and quantified demyelination and axonal loss at different stages of relapsing-remitting EAE in Biozzi ABH mice and showed that a loss of axons occurs independently from demyelination. Our results also demonstrate that although first triggered by immune attack, axon loss continues during remission when there is less inflammation.

**MATERIALS AND METHODS**

**Animals**

Biozzi ABH mice, originally supplied by Dr David Baker, Institute of Neurology, London, UK, were bred in-house under standard conditions of 12-hour light/dark cycle and fed ad lib. All experiments were conducted with the approval of the Research Animal Resources Center of the University of Wisconsin.

**Induction and Scoring of EAE**

Experimental autoimmune encephalomyelitis was induced in 8- to 12-week-old male and female Biozzi ABH mice by subcutaneous injection into the flank of syngeneic spinal cord homogenate emulsified in complete Freund adjuvant (Sigma-Aldrich, St Louis, MO) on days 0 and 7, as described previously (17). Each animal received 1 mg of spinal cord homogenate (a gift from Dr David Baker) and 60 μg mycobacterium (Mycobacterium tuberculosis H37Ra, Mycobacterium butyricum [4:1]; Difco, Lawrence, KS) per injection. Body weight and clinical signs were assessed daily as previously described (17, 18) using the following scoring system: 0, normal; 1, loss of tail tone; 2, impaired righting reflex; 3, partial hind limb paralysis, with 1 limb affected; 4, complete hind limb paralysis, with both limbs affected; 5, moribund. Remission from the active phase of disease was defined as the resolution of clinical paralysis, weight gain, and stabilization of the neurological deficit (17, 18). A relapse was defined as a change in clinical score of at least 1 point, together with developing of paresis (typically score 3 or above) associated with weight loss. Results are shown as the mean daily clinical scores ± SEM and mean maximum clinical scores; in some instances, average scores were calculated for a given disease period resulting in a measure of the length of time an animal had spent with active clinical disease.

**Tissue Sampling and Hematoxylin and Eosin Staining**

Spinal cords from normal, adjuvant-only controls (n = 6) and spinal cord homogenate-immunized animals were analyzed at various times postimmunization (p.i.). Tissues were sampled on day 12 p.i. (4 days before the median day of onset; n = 6), day 14 p.i. corresponding to the preclinical disease associated with weight loss (n = 7), during the initial acute paralytic attack (around 19 days p.i.; n = 12), and during the first remission around 28 days p.i. (n = 7). The pathological features of the mice during the first relapse, which occurs at around day 35 p.i., were reported previously (17) and were not studied here. Tissue was analyzed from the second and third relapses (around 58 and 75 days p.i., respectively; both n = 7). Briefly, after transcardiac perfusion with Ringer solution followed by perfusion with 4% paraformaldehyde in Ringer solution, the spinal columns were dissected and postfixed for 18 hours with paraformaldehyde. Tissues were then divided into several groups for further processing. In the first group (n = 4), the whole spinal cords were embedded in paraffin and 5-μm sections were cut and stained with hematoxylin and eosin (H&E). In the second group (n = 3), spinal cords were divided into 3 sections corresponding to cervical, thoracic, and lumbar areas. This tissue was cut sagittally and embedded in paraffin for H&E staining. In the last group, the spinal cords were divided into 16 blocks corresponding to every third vertebral segment. Alternate blocks were postfixed in 2.5% glutaraldehyde in Ringer solution and embedded in resin from which semithin 1-μm sections were cut and stained with toluidine blue. The remaining blocks were cut on a standard microtome for H&E staining. The brains were analyzed in some animals, but they showed limited pathological changes, as has been reported previously in this EAE model.

**Tissue Analysis**

Tissue analysis was performed using either the MetaVue software package (Molecular Devices, Inc, Sunnyvale, CA) and Nikon Microphot FXA microscope (Nikon Inc, Melville, NY; quantitative imaging) or the Bioquant software package (Bioquant Image Analysis Corp, Nashville, TN) and a Nikon Eclipse E800M microscope (qualitative analyses), each coupled with a high-resolution digital video camera.

**Quantification of Axonal Loss**

Analyses were performed on toluidine blue–stained semithin sections. A 2.5 × (objective lens) image of the cross-section of the spinal cord at thoracic vertebra level T4 was acquired by computer and used to position a fan-shaped region of interest with 3 fronds used as the perimeter of the sampling area (Fig. 1). The region was positioned with the

---

**References:**

center on the central canal with 1 frond bisecting the dorsal column. This sampling technique was used to account for the difference in fiber density between the center and outer edges of the spinal cord (19) and effectively sampled fibers in the dorsal column and at 2 ventrolateral sites. A threshold was applied within this region of interest that corresponded to the axoplasm of each axon; this threshold level was kept constant between sections. Nonaxonal structures such as astrocytic processes and oligodendrocyte cell bodies were excluded using size and shape filters and direct visual analysis. Fibers in contact with the edge of the region of interest were also discarded, and remaining axoplasm areas were counted automatically at 100× magnification. Axoplasm area measurements from all sampling sites were summed to give an overall figure for axon loss. The technique identified only axons with intact myelin. Data of axon count and axoplasm overall figure for axon loss. The technique identified only axons with intact myelin. Data of axon count and axoplasm area were compared and not found to be significantly different (not shown); therefore, axon count data were used. These data were compared with manually counted axon numbers from the same section (n = 3 sections) and were not found to be significantly different (not shown).

Quantification of Demyelination

Demyelinated axons were counted in the same regions of toluidine blue–stained semithin sections identified during axon quantification. Because the automatic threshold technique was not sufficiently specific for demyelinated axons, these were counted by the observers with the same rules as described above for intact axons.

Infiltration Load

Infiltration of peripheral immune cells into the CNS was estimated in the acute phase of disease in H&E-stained sections. A 4× (objective lens) image of the cross-section of the spinal cord at each vertebral level was acquired by computer. Hematoxylin-stained nuclei within cells were then identified by color, and a threshold corresponding to this staining was applied to the image. The pixel area of stained tissue was recorded and expressed as a percentage of the total cord area.

Lymphocyte Isolation From the Spinal Cord

Mice were anesthetized with a ketamine and xylazine mixture (1:2) and perfused transcardially with PBS. Spleen and spinal cord were removed, weighed, and put on ice in Hank buffered salt solution (Cellgro Mediatech, Herndon, VA). The spleen was homogenized between frosted glass slides, and the resultant cell suspension was pelleted by centrifugation. Red blood cells from the spleen were lysed in ammonium chloride potassium lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM EDTA, pH 7.3), and the remaining cells were washed 3 times with Hank buffered salt solution and counted. To isolate lymphocytes from the spinal cord, the tissues were minced, transferred to Medicon inserts, and ground in a MediMachine (Becton Dickinson, Mountain View, CA) for 30 seconds. The cell suspension was washed with Hank buffered salt solution, and cells were resuspended in 50% Percoll (Pharmacia, Piscataway, NY) and carefully overlaid with 30% Percoll. The gradient was centrifuged at 2,250 × g for 30 minutes at 4°C. The interface was collected and washed once for further analysis.

Flow Cytometry Analyses

Splenocytes and mononuclear cells isolated from the spinal cord (1 × 106/50 μL) were incubated in fluorescence activated cell sorting buffer (PBS containing 1% bovine serum albumin) with fluochrome-conjugated antibodies and 2.4G2.1 (Fc-block) for 30 minutes at 4°C and then washed. Cells were stained with anti-CD4 (clone RM4-5), anti-CD8 (clone 53.6–7), anti-CD45 (clone 30-F11) antibody (all from BD Pharmingen, San Diego, CA), and anti-CD11b antibody purified from the supernatant of clone M1/70 (American Type Culture Collection, Manassas, VA) conjugated with Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) using an Alexa Fluor 488 monoclonal antibody labeling kit (Invitrogen, Carlsbad, CA). Stained cells were analyzed using

FIGURE 1. Axon counting methods. (Left) After background correction, a fan-shaped region of interest was placed onto the section at 4×, with 1 frond bisecting the dorsal columns. This area was used as the sampling area. (Right) The objective was changed to 100× and the fan redrawn automatically by the computer corresponding to the same region of interest. A threshold was generated encompassing all axoplasmic area (red area); this is accomplished because the axoplasm is consistently lighter in color than other areas, particularly myelin. After user- and computer-derived exclusion of artifacts, the total axoplasmic area was calculated automatically and the total measured area of axoplasm summed. The same threshold was applied to each image.
a dual-laser FACSCalibur (Becton Dickinson, Franklin Lakes, NJ), and analyzed with the FlowJo software (TreeStar, Ashland, OR).

Statistical Analysis
All data were analyzed using 1-way analysis of variance with the Holm-Sidak posttest using the SigmaStat software (Systat Software, Inc, San Jose, CA). Correlations were performed with the Spearman rank order correlation test for nonnormally distributed data using the SigmaStat software.

RESULTS
ABH Mice Accumulate Clinical Deficits Over a Series of Relapses
ABH mice immunized with spinal cord homogenate to induce EAE were followed for 95 days to assess the disease during an extended period. There was a high incidence of disease (95%; n = 74) with a mean ± SD onset of clinical signs 16 ± 5.3 days p.i. As shown in Figure 2A, weight loss preceded the onset of disease and also occurred 1 to 2 days before each relapse, making this parameter a valuable predictor of disease onset and future relapses. Approximately 95% of animals developed complete hind limb paralysis (score 4) as reflected in Figure 2A, although the peak scores were slightly reduced as a result of the asynchronous onset of clinical signs. Eleven percent of mice that reached a grade 4 in the initial attack died or had to be euthanized due to exceeding the accepted limits of disease severity, such as becoming moribund and unable to feed; these animals were not included in the study. Active disease presented clinically as paralysis of the hind limbs and tail, but additional adverse pathological signs were also observed, most notably priapism in males and a failure to control urination and defecation in both males and females. We did not observe significant differences in the clinical course of disease between males and females, however. Eighty-four percent of animals developed a relapsing-remitting disease course, and only 16% exhibited a monophasic disease course with a single episode of paralysis followed by recovery with a variable degree of residual paresis. Animals initially remitted nearly completely after the acute phase of paralysis but demonstrated some residual tail atony; however, an increase in residual neurological deficit was observed after subsequent relapses (Fig. 2A). Active paralytic disease typically lasted 6 to 9 days followed by 15 to 18 days of clinical remission. Relapses and remissions were concurrent with weight loss and gain, respectively (Fig. 2A). At approximately 65 to 80 days p.i., after approximately 3 relapses, the mice entered a chronic phase of disease in which they did not recover from either severe paresis or paralysis.

Mononuclear Cell Infiltration Load Accumulates in the Thoracic and Lumbar Spinal Cord
Analysis of the cross-sectional area of the spinal cord during the acute phase of disease showed no evidence of a significant change in volume of the tissue suggestive of edema or atrophy. Analysis of cord from C1 to L11 levels

FIGURE 2. Disease course and cellular infiltration of experimental autoimmune encephalomyelitis (EAE) in ABH mice. (A) The relapsing-remitting EAE course in ABH mice is illustrated by the average disease scores (black line, left axis). There is near-complete recovery from clinical signs after the acute stage and a residual functional deficit after each relapse. After 3 remissions, the animals enter a chronic plateau disease phase in which they do not recover from paralysis. Weight loss occurs before an upcoming relapse in this model (gray bars, right axis). (B) Quantification of cellular infiltration in the acute phase of disease from hematoxylin and eosin (H&E)-stained serial sections through the length of the spinal cord (n = 4). Hematoxylin-positive staining is higher than control in the thoracic and lumbar but not the cervical spinal cord in ABH EAE, indicating infiltration of cells from the periphery in these lower cord levels. Yellow bars = control; red bars = EAE mice. (C) Sagittal section of part of the thoracic spinal cord shows multiple scattered inflammatory foci (yellow arrows). Original magnification: H&E, 20×. Inset diagram the plane of section.
demonstrated that areas of cell infiltration were particularly concentrated in the thoracic and lumbar cord with increases further down the cord to the caudal lumbar segments (Fig. 2B). The sagittal sections of spinal cords demonstrated typical perivascular inflammatory foci (Fig. 2C).

To analyze the temporal changes in the cellular composition in the spinal cord during EAE, mononuclear cells were isolated from spinal cords, and the frequencies of CD4+ T cells, CD8+ T cells, microglia (CD45intCD11b+), and macrophages (CD45highCD11b+) determined using flow cytometry. Microglia and macrophages were distinguished by the differential expression of CD45 in CD11b+ cells (20). CD4+ T cells were the predominant subset of invading cells at the acute peak of disease, with a marked paucity of CD8+ cells (Fig. 3A). During remission, the numbers of CD4+ T cells in the spinal cord declined and were indistinguishable from background levels.

**FIGURE 3.** Macrophages and CD4+ T cells are the predominant inflammatory cells in the spinal cord. (A) Single-cell suspensions prepared from the spinal cord were stained with anti-CD4 and anti-CD8, anti-CD45, and anti-CD11b antibodies to determine the subsets of infiltrating T cells or microglia (CD45intCD11b+) and infiltrating macrophages (CD45highCD11b+) populations at the indicated time points. Representative dot plots show expression of CD4 and CD8 or CD11b and CD45 molecules on cells from the spinal cord. Numbers represent the frequency in the gated populations. (B) Mean data are presented for the numbers of CD4 and CD8 T cells or microglia and macrophages. The numbers of isolated cells per gram of tissue are presented to normalize isolated cell numbers from spinal cord samples of various sizes. The numbers of infiltrating CD4 cells are significantly increased in the acute phase and the levels abated in remission. Macrophage infiltration occurs before the onset of clinical signs, preceding microglial activation and CD4+ cell influx. Data represents SD of 3 independent experiments (n = 9).
from those in naive mice. In this model, macrophage (CD45<sup>high</sup>CD11b<sup>+</sup>) infiltration preceded the accumulation of CD4<sup>+</sup> T cells and remained highly elevated in the spinal cord during the acute phase of disease (Fig. 3B). The numbers of CD45<sup>int</sup>CD11b<sup>+</sup> microglia increased in parallel, indicating that the proliferation of these cells occurred before disease onset and in the acute phase of EAE (21). During remission, both macrophage and microglia levels were reduced to those observed before the onset of disease.

Axonal Loss Is Predominantly Subpial and Increases With Disease Duration

Axonal damage and loss, as assessed by quantitative analyses of toluidine blue-stained semithin sections, were concentrated in the subpial zone of the spinal cord. Axonal loss was first evident at the acute disease stage (Figs. 4A–F) before notable demyelination was detected, suggesting that at this stage of disease, inflammation may play a key role in axonal damage. The extent of axon damage in the subpial zone increased with each subsequent attack, with a concurrent decrease in axon numbers. At the L4 segment, the dorsal column tended to exhibit less damage compared with the lateral and ventral subpial regions, but lesion location was variable among different animals. Analysis of toluidine blue-stained semithin sections from mice at different disease stages revealed that axonal loss occurs in a step-wise fashion, increasing after each relapse (Fig. 5A). The extent of axon loss correlated significantly with the number of relapses and, perhaps more importantly, with the cumulative clinical score of the animal to that point (Fig. 5B; Spearman correlation coefficient: \(-0.467, h = 0.0140, n = 27\)). This indicates that axon number is directly dependent on the duration of active disease.

**FIGURE 4.** Progressive subpial damage extends into the spinal cord parenchyma. (A–D) Toluidine blue-stained sections demonstrate ongoing white matter disease and axonal loss in the dorsolateral columns. Sections from control (A), acute stage (B), and second (C) and third relapses (D). White matter abnormalities increase with time. (E, F) Axonal degeneration deep to a rim of subpial abnormality at the second relapse (E, from area marked with arrow in C). At the third relapse (area from D<sup>*</sup> shown in high power in F), there are only a few demyelinated axons (arrows) remaining in the area of axonal loss and marked macrophage infiltration. Original magnifications: (A–D) 200 x; (E) 900 x; (F) 1,250 x.
disease. During the acute phase of disease, axon number was reduced by approximately 20%, although this did not reach statistical significance. The reductions continued during remission with 40% axon loss. At the chronic stage of EAE, after the third relapse, 66% of the axons in the dorsal column of the lumbar region were lost; this was associated with considerable neurological deficit.

Demyelination and Remyelination Persist Into the Chronic Stage of Disease

During the relapsing course of disease, areas of demyelination became more evident in the cervical dorsal columns compared with lumbar regions. Larger areas of damage were seen more commonly at the third relapse (Fig. 6B), although there were occasional mice with large areas of myelin and axon damage at earlier time points. These lesions exhibited mixed pathological features, with demyelination, remyelination, axonal loss, lymphocyte and macrophage infiltration, and vacuoles present within the same lesion area (Figs. 6A–G). Importantly, axon numbers did not correlate with the demyelinated axon count during the course of the disease when compared with the Spearman rank correlation test (data not shown). Oligodendrocyte-mediated remyelination was identified in many lesions; Schwann cells remyelinated a small number of axons adjacent to the nerve roots. Interestingly, remyelination was noted in areas of profound macrophage/microglial activity (Fig. 6F). Demyelinated axons were frequent adjacent to vessels cuff ed by mononuclear cells (Fig. 6E). Chronic paralysis usually developed after 3 relapses, and lesions at the chronic stage of EAE contained numerous macrophages and microglia. Many axons survived until this stage of disease along with fewer remyelinated axons (Fig. 6G). The numbers of demyelinated axons peaked at the second relapse and fell again in the chronically affected animals (third relapse). This decrease in demyelinated axons at the late time point is likely to be due to the ongoing axon loss by this stage in the disease and not to an increase in remyelination because an increase in the latter was not apparent at this stage.

DISCUSSION

This study examines the long-term clinical course and histopathologic changes in the spinal cord during EAE induced with spinal cord homogenate immunization in Biozzi ABH mice. The relapsing-remitting disease followed by a chronic-progressive course reported here is consistent with previous reports (17, 18, 22, 23). Inflammation and acute axon loss are the predominant changes in the spinal cord early in the course, followed by widespread demyelination, remyelination, and chronic axonal injury and loss. The severity of axon loss correlates well with the duration of active disease. By the third relapse, most mice become severely paraparetic or paraplegic with marked loss of axons and chronic demyelination. CD4+ T-cell infiltrates are predominant in the early phase, and their numbers decline during remissions, as do the numbers of macrophages and microglia. These findings define a novel EAE model that faithfully replicates many of the clinical, pathological, and immunological events seen

FIGURE 5. Axons loss is progressive in experimental autoimmune encephalomyelitis (EAE) in ABH mice. (A) Axons were counted in toluidine blue-stained sections as described in Materials and Methods section. There is progressive axon loss at each stage after the acute attack; loss becomes significant at the first remission (1-way analysis of variance with Holm-Sidak post-test). (B) Axon numbers correlated significantly with cumulative scores (linear regression). (C) Quantification of demyelinated axons reveals that demyelination follows the acute stage and increases over subsequent relapses. At the late chronic stage of disease, demyelinated axons are a less prominent feature of the disease, possibly because extensive axon loss has already occurred.
in relapsing-remitting MS with conversion to secondary progressive disease.

The wide variety of EAE models available provides many opportunities for studying various aspects of the pathogenesis and therapy of this disease model that could be relevant to MS (10, 24). Defining a model with a clinical course similar to relapsing-remitting MS has, however, proved to be challenging because many EAE models show only minimal clinical improvement (remission) between relapses or have a truly chronic and often progressive course. Although the overall time course of the disease in Biozzi ABH mice is compressed and much shorter than in MS, it nonetheless seems to match the natural history of the human disease and be better than other rodent models. Experimental autoimmune encephalomyelitis has also been induced in Biozzi ABH mice using immunization of the extracellular domain of the MOG peptide (residues 1-125), resulting in a relapsing-remitting course, but the remissions are much less prominent than those immunized with spinal cord homogenate (25). In contrast, induction of disease with MOG peptide 8-21 also

**FIGURE 6.** Lesions display mixed histopathologic features. (A, B) Posterior columns. Lesions generally expanded in size from the second (A) to the third relapse (B). At the second relapse (A), numerous demyelinated axons were seen in subpial areas but not deeper in the dorsal columns. At the third relapse, only scattered demyelinated axons were present (B). (C, D) Lateral columns. Large areas of demyelination were common in the lateral columns at the second (C) and, in some mice, at the third relapse (D) where there was also evidence of axon degeneration (arrows). (E, G) Ventral column. Demyelinated axons are frequent adjacent to areas of blood vessel inflammatory cell cuffs. Demyelinated axons (arrow) are shown at higher power in the inset. Despite marked foamy macrophage infiltration in and demyelination the second relapse, there are 4 thinly myelinated, remyelinated axons (arrows) (F). At later stages (third relapse), demyelination and remyelination (R) adjacent to axons undergoing degeneration (arrows) are seen (G). Original magnifications: (A, B) 125×; (C, D) 1,000×; (E) 600×, (inset) 2,000×; (F) 1,200×; (G) 1,500×.
induces a clear relapsing-remitting disease (26), which is less well characterized than this model, whereas MOG 33-55 induces a severe, chronic disease (15). Although most active EAE immunization protocols result in inflammation and variable tissue changes in the spinal cord, there is usually little evidence of inflammation in the brain or optic nerves. Interestingly, immunization of Biozzi ABH mice with an optic nerve homogenate results in reproducible lesions in the optic nerve (27). In the present study, CD4+ cells were the dominant T-cell phenotype in acute and relapsing stages of disease, with a paucity of CD8+ T cells, a finding consistent with previous studies. This latter phenotype seems to be predominant in MS lesions and can contribute to pathology in EAE (21, 28-31). Based on previous studies, it is expected that Biozzi ABH mice respond with high titer antibody production upon immunization (32). The paucity of detectable CD8+ T-cell infiltration activity in this model suggests that when CNS autoimmune diseases are associated with B-cell responses and higher immunoglobulin production, CD4+ T cells dominate the disease.

The loss of axons in MS is clearly critical to neurological function and the long-term prognosis in patients. We show for the first time that each episode of disease is associated with a stepwise loss of axons in the spinal cord in the Biozzi ABH mouse model. The greatest degree of axon loss occurs in the acute stage where there is a 20% loss of axons; a similar loss occurs during the first remission, despite recovery to a near-normal clinical state. Axonal degeneration is most evident in subpial zones of the ventral and lateral columns of the spinal cord. Similarly in MS, acute axonal death is most frequently seen in early disease and decreases with time (33). In the early stages of EAE in Biozzi mice, recovery may be due to 2 mechanisms, that is, the considerable degree of plasticity within the CNS that allows functional recovery and remyelination of the few axons that are demyelinated in the first attack. Importantly, we found that demyelination during acute disease was infrequent and hence was not likely to account for the extensive axon loss that occurs at this time.

Loss of axons in MS models associated with inflammation and related to disabilities has been reported previously in EAE. In chronic EAE, axon loss has been well documented (8, 34, 35). Chronic, relapsing-remitting EAE may, however, offer a better opportunity to understand what happens to axons in early and secondary progressive MS. Wujek et al (36) produced chronic, relapsing disease in SWXJ mice immunized with myelin proteolipid protein. Affected mice developed a chronic disease course with up to 5 relapses, during which time axon number and inflammatory cell infiltrate were quantitated early and late in disease when a fixed neurological deficit had been achieved. The authors found that inflammation rather than axon loss correlated with clinical disability early in disease but not at later stages when the number of relapses and axon loss correlated with neurological impairment. Papadopoulos et al (7) compared 2 models of EAE in the Dark Agouti rat using different doses of MOG and found that the lower dose of MOG produced a chronic progressive disease, whereas the higher dose produced a relapsing-remitting disease with up to 6 relapses. Axons were counted in the dorsal and lateral columns, and demyelinated axons were also quantitated. They concluded that 1) axon loss showed a strong correlation with worsening clinical deficit; 2) there was no correlation of inflammation or demyelination with axon loss; 3) the greater the number of relapses, the greater degree of axon loss, such that a single relapse could result in a marked loss of axons; and 4) small diameter axons were the primary target. They also noted that at the end stage of disease, inflammation had nearly resolved and there was no evidence of active demyelination.

Each of these models, including the Biozzi ABH model reported here, implicates axon loss as a cause of the chronic neurological disability. There are similarities and differences between each model, but they are complementary in many respects. The degrees of axon loss in our study and that of Wujek et al (36) are similar (i.e. up to 60%), whereas there was a maximum of 32% in the study of Papadopoulos et al (7). The latter authors noted that quantitation of axons is potentially more accurate in 1-μm plastic sections than in immunolabeled or silver-stained tissue sections. The range of loss of axons quantitated in these 3 models indicates considerable variability in the loss necessary to produce chronic motor deficits such as paraplegia. The percentage of axons that must be lost for motor function to be permanently impaired is not clear from studies of axon loss in the spinal cord of MS patients, and loss of axons may not correlate with disease duration (37). In the study of Bjartmar et al (38), axon loss in the spinal cord of patients paralyzed at death varied from 45% to 84%. Presumably, this loss involves descending motor tracts. Likewise in EAE, axon loss that affects only motor tracts would result in an apparent lesser overall loss of axons, such as the findings in Reference (7). In the 3 EAE studies, inflammation lessened over time and the number of relapses correlated strongly with axon loss, and we showed that the amount of time spent in active disease also correlated strongly with the number of axons lost. The degree of demyelination was quantitated in our study and by Papadopoulos et al (7) and seemed to correlate poorly with axon loss. Similarly in MS, a study of plaque load (i.e. areas of demyelination) suggested that axon loss in the spinal cord occurred independently of inflammation-induced demyelination (39). In Biozzi mice in the present study, there was persistent demyelination, suggesting that the current model has a greater similarity to chronic MS in which demyelinated plaques are frequently adjacent to remyelinated axons in shadow plaques.

In the model reported here, demyelination first became evident after the acute attack and peaked during the second relapse, occurring apparently independent of axonal loss. In active demyelination, at the first or second relapse, large plaques of demyelinated axons associated with lipid-filled macrophages were seen as well as scattered individual or small groups of demyelinated axons. In some cases, there was almost complete axon loss adjacent to these plaques of demyelination. In areas of active macrophage activity (i.e. acute demyelination), there was frequently evidence of active oligodendrocyte remyelination (40). This underscores the role that inflammation may play in remyelination and that macrophages or activated microglia may have dual roles in...
tissue degradation and repair (41). In Biozzi AB mice, remyelination was predominantly carried out by oligodendrocytes and not Schwann cells, although this was not quantitated. It seemed that demyelination could persist for long periods as judged by the presence of plaques of demyelination that had little evidence of active macrophage involvement (Fig. 6). It is of course difficult to determine exactly the time point at which demyelination occurred in any plaque.

In summary, the model of EAE described here provides a very useful system in which all the pathological hallmarks of chronic MS are present, thereby allowing the testing of therapies that might promote remyelination of chronically demyelinated axons and new neuroprotective agents. It matches well the disease progression seen most commonly in MS, with a relapsing-remitting disease advancing to a secondary progressive phase. The key will be to perform appropriately designed experiments in which myelin repair and neuroprotection can be quantitated and in which the development of chronic disability is prevented.

REFERENCES
7. Papadopoulos D, Plam-Dinh D, Reynolds R. Axon loss is responsible for chronic neurological deficit following inflammatory demyelination in the rat. Exp Neurol 2006;197:373–85
10. Steinmann L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. Ann Neurol 2006;60:12–21