Axon Loss in the Spinal Cord Determines Permanent Neurological Disability in an Animal Model of Multiple Sclerosis

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Abstract. Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Most patients undergo an initial relapsing-remitting (RR-MS) course that transforms into a relentless neurodegenerative disorder, termed secondary progressive (SP)-MS. Reversible inflammation and demyelination account readily for the pattern of RR-MS but provide an unsatisfactory explanation for irreversible decline in SP-MS. Axon loss is thought to be responsible for progressive, non-remitting neurological disability during SP-MS. There is considerable potential for neuroprotective therapies in MS, but their application awaits animal models in which axonal loss correlates with permanent neurological disability. In this report, we describe quantitative immunohistochemical methods that correlate inflammation and axonal loss with neurological disability in chronic-relapsing experimental autoimmune encephalomyelitis (EAE). At first attack, CNS inflammation, but not axon loss, correlated with the degree of neurological disability. In contrast, fixed neurological impairment in chronic EAE correlated with axon loss that, in turn, correlated with the number of symptomatic attacks. As proposed for MS, these observations imply a causal relationship between inflammation, axon loss, and irreversible neurological disability. This chronic-relapsing EAE model provides an excellent platform for 2 critical objectives: investigating mechanisms of axon loss and evaluating efficacy of neuroprotective therapies.

Key Words: Axonal loss; Experimental autoimmune encephalomyelitis; Inflammation; Multiple sclerosis.

INTRODUCTION

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the CNS (1) and the most common cause of non-traumatic primary neurological disability in young adults. Most patients undergo an initial relapsing-remitting (RR-MS) phase that transforms into a continuously secondary progressive disease (SP-MS). The reversible nature of inflammation and demyelination can explain the pathogenesis of RR-MS (2), but does not provide a satisfactory explanation for the transition to chronic non-remitting neurodegeneration in SP-MS. In this regard, recent studies have indicated that axon loss begins early in RR-MS, continues throughout the disease course (3–5) and often reaches a threshold that is responsible for permanent neurological disability (6). Importantly, axon loss in the spinal cord (6) and spinal cord atrophy (7, 8) on magnetic resonance imaging (MRI) exhibit the most impressive correlations with paralysis and loss of ambulation.

Disease mechanisms relevant to MS have been studied extensively in the animal model experimental autoimmune encephalomyelitis (EAE). EAE is induced by immunization of animals with myelin or myelin protein peptides. The susceptibility and pathogenesis of EAE vary dramatically among different species and strains of animals, and are significantly affected by the choice of immunogen and immunization technique (1, 9). Genetic background influences EAE susceptibility and disease progression. Elegant back-crossing experiments between susceptible and resistant strains have identified both major histocompatibility complex (MHC) genes and non-MHC genes as important factors that regulate the expression and severity of EAE in mice (10–12). In SWXJ mice immunized with proteolipid protein (PLP) peptides and in marmosets immunized with human white matter, the disease is chronic and characterized by perivascular lymphocytic and monocytic infiltrates and by demyelination (13–15). In Lewis rats immunized with myelin basic protein (MBP) or in outbred rhesus macaques immunized with bovine brain homogenate, EAE is a hyperacute disease consisting of neutrophil and T-cell inflammation and limited demyelination (9, 16). Despite their varied time courses and pathological bases, neurological disability in each of these models is typically severe and often fatal. The dissociation between inflammation and demyelination in EAE models has been recognized for decades, raising the possibility that neurological disability can be caused by diverse mechanisms. One potential way to understand the role of inflammation, demyelination, and axon loss as they relate to neurological disability is to segregate them in animal models.

Study of EAE models has focused on pathogenic mechanisms relating to disease susceptibility, inflammation, and demyelination. While the importance of axonal loss in the pathogenesis of MS has been recognized (3–5), axonal pathology in EAE has not been studied extensively. Acute axonal pathology correlates with the presence of macrophages in a Lewis rat EAE model (17).
The relationship between axonal pathology or loss and acute or chronic neurological disability has not been reported in this or other EAE models.

Female SWXJ (H-2\(^s\)) mice immunized with the p139–151 peptide of myelin proteolipid protein (PLP) develop relapsing-remitting chronic EAE characterized initially by intermittent attacks of reversible neurological impairment, with a late plateau of sustained functional disability (13). The predominant site of inflammatory tissue injury in this model is the spinal cord and the clinical rating scale used in EAE (as in MS) emphasizes spinal cord function. Therefore, the clinical, histological, and temporal pattern of disease in this EAE model mimic the most common profile observed in MS. We used this relapsing-remitting EAE mouse model (13) to correlate spinal cord inflammation and axon loss with reversible and irreversible neurological disability.

**MATERIALS AND METHODS**

**Induction of EAE**

SWXJ (H-2\(^s\)) mice were produced by mating SWR/J (H-2\(^s\)) females with SJL/J (H-2\(^s\)) males. All protocols for animal research were approved by the Cleveland Clinic Foundation Animal Research Committee, in compliance with the Public Health Service policy on humane care and use of laboratory animals. To induce chronic-relapsing EAE (13), adult female mice were injected subcutaneously on Day 0 with a mixture of 100 nanomoles PLP peptide p139–151 and 400 micrograms *Mycobacterium tuberculosis* H37RA in complete Freund’s adjuvant. These mice were also injected intravenously on Day 0 and Day 3, with 2 \(\times\) 10\(^3\) to 4 \(\times\) 10\(^3\) *Bordetella pertussis* bacilli (Michigan Department of Public Health, Lansing, MI). Control mice received the same, but without the PLP peptide.

**Monitoring Clinical EAE**

Mice were weighed and examined daily for neurological signs according to previously published criteria (13): 0 = no observable symptoms; 1 = flaccid tail; 2 = poor righting reflex; 3 = clumsy gait; 4 = limp paralysis; 5 = moribund. Mice were killed 5 days after the first neurological attack, defined as a concomitant increase in clinical score and loss of body weight, or at the chronic stage of EAE (3 months post-immunization). The peak severity of acute EAE was defined as the highest score achieved by each mouse during the first 5 days of the first attack. In virtually every case, this score was maintained for at least 2 consecutive days. Clinical severity during the plateau phase of chronic non-remitting EAE was established as a disability score that did not change for 1 wk, at least 45 days post-immunization.

**Light Microscopy and Immunohistochemistry**

Mice were deeply anesthetized and perfused through the heart with 4% paraformaldehyde in 0.08 M phosphate buffer. The cervical and lumbar spinal cord segments were removed, post-fixed, and cryoprotected in 20% glycerol. Free-floating cross-sections (30-\(\mu\)m-thick) were cut, placed in cryostorage solution (1% polyvinylpyrrolidone-40, 30% ethylene glycol, and 30% sucrose in 0.2 M phosphate buffer) and stored at \(-20^\circ\)C. For immunostaining, sections were rinsed with phosphate-buffered saline, incubated in tris-buffered saline with 0.25% Triton X-100 plus 0.3% hydrogen peroxide, and incubated with a primary antibody. The antibodies were a rabbit polyclonal antibody against the 200 kDa neurofilament protein (Serotec, Raleigh, NC; AHP245; diluted = 1:10,000); a rat monoclonal antibody against CD45 (Serotec; MCA1388; diluted = 1:8,000); a rabbit polyclonal antibody against CD3 (Dako, Bedford, MA; diluted 1:8,000). Sections were rinsed, incubated in biotinylated secondary antibodies (Vector Labs, Burlingame, CA), rinsed, incubated in an avidin-biotin peroxidase complex (Vector Labs), and visualized with a nickel-intensified diaminobenzidine reaction. Tissue sections were mounted on microscope slides and coverslipped.

**Electron Microscopy**

Mice were deeply anesthetized and perfused with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.08 M phosphate buffer. Spinal cord segments were dissected out, post-fixed, and embedded in Epon. Ultrathin sections of white matter were cut in the transverse plane, mounted on Formvar coated grids, and photographed in a Philips CM100 electron microscope.

**Morphometry**

To determine the extent of inflammation in control and EAE spinal cord, the level of CD45 immunoreactivity (microglia, macrophages, monocytes, and lymphocytes) was quantified. Images at low magnification (5\(\times\) objective lens) of whole spinal cord were digitally photographed using a Leica DMR microscope fitted with an Optronics Magna Fire CCD color video camera and image acquisition system. Digital images were captured (using Adobe Photoshop 5.0 software; Adobe Systems Inc., San Jose, CA) and coded. Spinal cord area occupied by CD45 immunoreactivity was determined by measuring number of pixels above a set threshold value. Spinal cord area was measured (total pixels within spinal cord) and the percent area of CD45 immunoreactivity was calculated. To determine lymphocyte density, the numbers of lymphocytes were measured in white matter of spinal cord sections stained for CD3. With an ocular reticle, CD3-positive cells were counted at high magnification (100\(\times\) objective and 10\(\times\) ocular lens). For each animal, a total area of 0.9 mm\(^2\) was analyzed and values were calculated as cells per mm\(^2\). To determine the extent of axon loss, axons were counted in anti-neurofilament stained spinal cord sections of EAE and control mice. Digital images at high magnification (100\(\times\) objective lens) were captured in selected areas, as described above. Axon counts were made in a blinded fashion, using NIH Image computer software (version 1.61). Because axon density varied among the different spinal cord areas, the axonal number in each area of EAE spinal cord was expressed as a percent of mean control values in the same area. Since inflammatory tissue swelling is prominent in EAE, spinal cord areas were measured and used to normalize axonal densities.
**Statistical Analysis**

Of the mice killed at first attack of EAE, the number of mice analyzed was as follows: mice with clinical score of 2 (n = 4), mice with clinical score of 4 (n = 4), and control mice (n = 5). Of the mice killed at the chronic stage of EAE, 6 mice were analyzed from each level of clinical score (0, 2 and 4), plus a control group of 6 mice.

Data were analyzed with the Student t-test. Relationships between symptomatic attacks, clinical scores, and pathological alterations were evaluated by regression analysis. Specifically, the number of attacks experienced by each mouse was plotted against its final clinical score and extent of axonal loss. The correlation coefficient (r) and the significance level (p) for these 2 variables were calculated using the Spearman rank correlation test.

**RESULTS**

Quantitating Neurological Disability, Inflammation, and Axonal Pathology in First-Attack and Chronic Non-Remitting EAE

This study was designed to establish the relationship between neurological disability, inflammation, and axon loss in EAE. We selected 2 distinct phases of disease for examination: 1) peak severity of first attack; and 2) the plateau stage of chronic EAE, during which mice exhibited fixed neurological disability. These stages of EAE were used to provide surrogates for the relapsing-remitting (inflammatory) portion of MS and the secondary progressive (neurodegenerative) phase. The standard rating scale for EAE clinical severity (see Materials and Methods) monitors spinal cord function and was therefore appropriate for this study.

We determined inflammation intensity and axonal number in spinal cords of EAE and control mice. Inflammatory lesions in MS and EAE are characterized by infiltration of hematogenous lymphocytes and monocytes or macrophages, plus activation of resident microglia (18–22). Since all these cell types express the pan-leukocyte antigen CD45 (23), we used the spinal cord area occupied by CD45-positive cells as an indication of overall inflammatory activity. Because T lymphocytes are critical for inducing EAE and for regulating the activity of other inflammatory cells, we also measured CD3-positive cells in control and EAE spinal cord.

Axons were counted in 3 standardized fields (dorsal column; dorsal-lateral; ventral) from defined levels of both cervical (C3) and lumbar (L4) spinal cords. Images from selected areas (Fig. 2A) were digitally photographed, coded, and total axons were quantified in a blinded fashion. Because axon density varied in different regions of the spinal cord, the axonal numbers were expressed as a percent of the mean control value for the corresponding field at that level. Inflammatory swelling is prominent in the CNS tissues of acute EAE mice and varies according to clinical score. Therefore, axonal density was normalized to spinal cord area.

**Inflammation Determines Disability in Early-Stage EAE**

For analysis of pathological determinants of neurological signs during the first attack of EAE, mice with clinical scores of 2 and 4 were killed 5 days after onset of initial symptoms. In control mice, CD45 immunoreactivity was detected almost exclusively in resting microglia (Fig. 1A, E), which occupied 12%–13% of spinal cord area (Fig. 1D). At higher magnification these microglia were seen to possess small cell bodies and numerous cytoplasmic processes. In spinal cords of first-attack EAE mice, the density of CD45-positive cells increased dramatically (Fig. 1B, C). CD45-positive cells occupied 20% (cervical) and 34% (lumbar) of spinal cord area in EAE mice with clinical score = 2 and 45% (cervical) and 57% (lumbar) of spinal cord area in mice with clinical score = 4 (Fig. 1D). Inflammatory infiltrates were prominent in subpial white matter, along larger vessels penetrating from the pial surface, and at other locations in the white and gray matter (Fig. 1B, C). At higher magnification (Fig. 1F), many activated microglia were observed with short stubby cell processes; also, the extremely high density of inflammatory cells was readily apparent. These occurred throughout the cervical and lumbar spinal cord and were not restricted to areas of myelin loss. In control spinal cord, CD3-positive cells were rare (Fig. 1G, I). However, at the first attack of EAE symptoms, T lymphocyte density increased greatly (Fig. 1H) and was observed to be higher at increasing clinical scores (Fig. 1I). Importantly, there was a significant correlation (r = 0.76; p = 0.03) between clinical score and area occupied by CD45-positive cells in the cervical spinal cord (Fig. 1D). There was also a significant correlation between clinical score and CD3-positive cells (r = 0.94; p = 0.001).

Analysis of the lumbar spinal cord revealed a non-significant relationship between clinical score and inflammation (r = 0.65; p = 0.08). These data identified spinal cord inflammation as a major determinant of neurological disability during the first attack of EAE.

Since axonal transection occurs early in MS and is related to the degree of inflammation (3, 5), we determined the extent of axon loss associated with the first attack of EAE (Fig. 2). Remarkably, during the first attack of EAE, axonal density varied but did not appear different from control (Fig. 2B–D) and bore no relationship to clinical score (Fig. 2E). In individual mice, axon loss varied between 0% and 22%. Thus, axon loss may occur during the first attack but was not sufficient to produce neurological deficits as determined by the clinical severity score. These data support the hypothesis that inflammation is the principal determinant of reversible neurological disability during acute EAE.
Spinal cord inflammation correlates with reversible neurological disability during initial attack in EAE mice. A–C: CD45 immunoreactivity in transverse spinal cord sections from a control mouse (A) and EAE mice with clinical scores of 2 (B) and 4 (C). In control tissue, CD45 immunoreactivity is restricted to resting microglia. Five days after initial onset of disability, EAE spinal cord sections contained elevated CD45 immunoreactivity, with intensely stained foci of inflammatory cells around blood vessels and adjacent to the pial surface. D: The spinal cord area occupied by CD45 staining increased significantly as the clinical score increased (Spearman’s rank correlation test: $r = 0.76; p = *0.03$). E and F: Higher magnification of CD45 immunoreactivity in control (E) and EAE mouse with clinical score of 4 (F). In control (E), immunostained cells are primarily resting microglia, exhibiting small cell bodies and thin cell processes (arrow). EAE spinal cord contains high density of CD45-positive cells (F); activated microglia (arrow) are recognized by their short, thick cell processes. A high density of other inflammatory cells is also observed in EAE, especially surrounding blood vessels. G and H: CD3 immunoreactivity in control (G) and in EAE mouse, with clinical score of 4 (H). In control tissue, CD3-positive cells are scarce, whereas the density is greatly increased at first attack of EAE. These cells are particularly abundant in the vicinity of blood vessels. I: The density of CD3 cells increased significantly as the clinical score increased (Spearman’s rank correlation test: $r = 0.94; p = *0.001$). Scale bar: A–C = 200 μm; E–H = 50 μm.

Inflammation Does Not Correlate with Non-Remitting Disability During Chronic EAE

Spinal cord CD45 immunoreactivity was quantitated in control (Fig. 3A) and chronic EAE mice with stable clinical scores of 0, 2, and 4 (Fig. 3B–D). CD45 immunoreactivity (Fig. 3E) was modestly increased in cervical (11%) and lumbar (5%) cords from mice with clinical scores of 4. In all other spinal cord sections from chronic EAE mice, CD45 immunoreactivity was present at control levels. Inflammation was much lower at all clinical levels of chronic EAE, when compared to severe first-attack EAE (clinical score = 4; Fig. 1D). In a similar manner, CD3-positive cell density was increased somewhat at clinical scores = 2 and 4 (Fig. 3F), but were much lower than at first attack (Fig. 1I). The majority of CD45-positive cells in chronic EAE spinal cords were process-bearing microglia. Occasional small foci of intensely CD45-immunoreactive phagocytic macrophages and CD3-positive lymphocytes were detected in the spinal cords of chronic EAE mice. Inflammation, whether measured by CD45 or CD3, did not correlate with neurological disability at the chronic stage of EAE, as determined by regression analysis.

Axon Loss Determines Disability in Late-Stage EAE

In contrast to inflammation, increased axon loss correlated with increasing neurological disability in both the cervical and lumbar spinal cord of chronic EAE mice.
**Fig. 2.** At first attack, axon loss in spinal cord does not correlate with reversible neurological disability. A: Control spinal cord cross-section stained for myelin with anti-PLP (boxes represent areas from which axons were quantified). B-D: Neurofilament-positive axons in dorsolateral spinal cord from control (B) and EAE mice with clinical score of 2 (C) and 4 (D). The appearance of axons is similar among the 3 groups. E: Quantitative analysis of axon density in cervical and lumbar cord, expressed as percent axon loss relative to control tissue. Axonal density was similar in sections from control and EAE mice (two-tailed, unpaired Student t-test). Scale bar: A–D = 200 μm.

**Fig. 3.** Spinal cord inflammation does not correlate with permanent neurological disability in mice with chronic EAE. A–D: CD45 immunoreactivity in transverse spinal cord sections from control mouse (A) and EAE mice with clinical scores of 0 (B), 2 (C), and 4 (D). In control tissue, CD45 immunoreactivity is restricted to resting microglia. Mice with EAE contain slightly elevated CD45 immunoreactivity, with a few small foci of inflammatory cells. Although some values were significantly different from control (*t-test, p < 0.05), there was no correlation between CD45 staining and clinical score, as determined by Spearman rank correlation test (E). F: CD3-positive cell density was significantly increased at clinical score = 2 and 4 (*t-test, p < 0.05), but no correlation was found between lymphocyte density and clinical score, as determined by Spearman rank correlation test. Scale bars: A–D = 200 μm.
Spinal cord axonal loss correlates with permanent neurological disability in mice with chronic EAE. A–D: Neurofilament-positive axons in dorsolateral region of spinal cord from control (A) and EAE mice with clinical scores of 0 (B), 2 (C), and 4 (D). Axonal loss increased with increasing clinical score. E: Quantitative analysis of axon density in cervical and lumbar cord, expressed as percent axon loss relative to control tissue. Axon loss significantly increased at each higher clinical score (Spearman’s rank correlation test; cervical cord: $r = 0.75; p = 0.0001$, and lumbar cord: $r = 0.63; p = 0.004$). Scale bars: A–D = 10 μm.

Fig. 4A–D. Mildly disabled chronic EAE mice (clinical score of 2) averaged 48% and 28% axon loss in cervical and lumbar cord. Axon loss in mice with non-remitting paralysis (clinical score of 4) averaged 59% and 43% in cervical and lumbar cords. Axon loss was highly correlated with clinical score (Fig. 4E) in both the cervical ($r = 0.75; p = 0.0001$) and the lumbar spinal cord ($r = 0.63; p = 0.004$). The quantity of axon loss experienced in the EAE mice approximates those (68% axon loss) recently described in spinal cord lesions of paralyzed MS patients (6). Examination of control (Fig. 5A) and EAE (Fig. 5B) spinal cords by electron microscopy confirmed abundant axon loss in chronic EAE mice and demonstrated myelin debris in macrophages. Spinal cord atrophy, which can be pronounced in SP-MS patients (7), was not detected in chronic EAE mice. As described in MS lesions (24), we found astrogliosis in regions of high axon loss associated with chronic EAE (not shown). These results support axon loss as a major determinant of neurological disability in chronic EAE mice.

Relapse Number Correlates with Axon Loss and Final Disease Severity

The number of symptomatic attacks experienced by chronic EAE mice ranged from 1 to 5. Regression analysis was used to investigate the relationship among attack number, fixed neurological disability, and axon loss. The extent of axonal loss significantly correlated with the number of attacks. This relationship was more robust for axon loss in the cervical cord (Fig. 6A, $r = 0.72; p = 0.0004$) than in the lumbar cord (Fig. 6B, $r = 0.45; p = 0.05$). As predicted from the relationship between attack number and axon loss, the number of relapses for each mouse was significantly related to the level of fixed neurological disability (Fig. 6C, $r = 0.59; p = 0.007$).

DISCUSSION

Most MS patients initially experience reversible functional impairment that eventually transforms into continuous neurodegeneration. It has been proposed that reversible neurological disability results from inflammatory brain lesions while axonal loss is the major cause of permanent disability (3, 6, 25, 26). We provide direct experimental support for this hypothesis in a chronic relapsing-remitting rodent model of MS. As described in MS, these EAE mice experienced different numbers of relapses and variations in acute and chronic neurological disability. Since most inflammatory lesions in these mice occur in the spinal cord (13), we correlated neurological disability with spinal cord inflammation and axonal loss at first attack and at end-stage disease. The severity of neurological disability at initial attack correlated with the extent of spinal cord inflammation while end-stage neurological disability correlated with axonal loss. Furthermore, axonal loss and end-stage disability correlated with the number of relapses, supporting a causal relationship between inflammatory attacks, axonal loss, and permanent neurological disability. A major goal of MS therapeutics is to stop or delay axonal loss and permanent neurological disability. The animal model described here mimics clinical and pathological aspects of MS and should prove useful in investigating inflammatory mechanisms of axonal transection and efficacy of neuroprotective therapies.

Previous studies have correlated various surrogate markers of inflammation, including numbers of perivascular cuffs and parenchymal leukocytes with disease severity in EAE models (27–29). Qualitatively, in a rat EAE model, T cells determined lesion topography and overall severity of inflammation, yet the clinical score
Fig. 5. Ultrastructural confirmation of axonal loss in chronic EAE spinal cord. A: Electron micrograph of normal myelinated axons in spinal cord white matter from a control mouse. B: Electron micrograph of spinal cord white matter from a mouse with EAE, illustrating severely reduced axon density, myelin debris and phagocytic macrophages with lipid vacuoles. Scale bars: A, B = 5 μm.

Fig. 6. The number of neurological attacks correlates with axon loss and chronic disease severity in mice with EAE. A and B: In both the cervical and lumbar cord, axon loss correlated with number of attacks (Spearman rank correlation test; \( r = 0.72; p = 0.0004 \), and \( r = 0.45; p = 0.05 \), respectively). C: In addition, the number of attacks correlated with final clinical score (Spearman correlation test, \( r = 0.59; p = 0.007 \)).

correlated best with the density of ED-1-positive cells (29). Since lymphocytes are critical for inducing EAE, whereas macrophages and microglia act as the effector cells, it is important to account for all cell types when relating inflammation to neurological disability. CD45, a pan-leukocyte marker, is expressed on lymphocytes, monocytes, macrophages, and microglia (23). In control mice, approximately 13% of spinal cord area is occupied by CD45-positive resting microglia and perivascular macrophages. In spinal cords from EAE mice with clinical score 2 and 4, the density of CD45-positive inflammatory cells was, respectively, twice and four times that found in control mice. Thus, the density of CD45 immunoreactivity is a reliable and reproducible surrogate...
marker for relating inflammation to neurological disability during initial attack in our EAE mice.

At first attack, the correlation of both CD45 and CD3 immunoreactivity with severity of clinical score demonstrates the importance of inflammation in generating reversible neurological disability, but does not address mechanism. One possibility is that edema, related to inflammatory vascular leakage, might reversibly compress spinal cord tissues (30). In support of this interpretation, the cross-sectional spinal cord area of our paralyzed mice was increased 9% over controls at first attack, but had returned to normal at end-stage disease. Additionally, activated inflammatory cells release cytokines and reactive oxygen species, which could potentially transiently impair neural function (31–33). In this regard, direct neurophysiological effects of cytokines have been documented in relevant model systems. Classic electrophysiological/pathological studies in normal rabbits demonstrated that intra-ocular injection of either IFN-γ or IL-1β resulted in reversible conduction delay that began within 1–3 h before histological inflammation and was related to effects on the vasculature (34, 35). Detailed clinical and immunological studies in MS patients that received a humanized anti-leukocyte monoclonal antibody showed that a transient treatment-related “cytokine storm” was associated with transitory recurrence of symptoms that arose from previously demyelinated lesions (36).

Prior quantitative analysis at first attack in this EAE model (37) detected an 8% decrease in myelin abundance relative to controls. Our qualitative analysis during the current study confirmed that demyelinated axons were not abundant at first attack or at end-stage (data not shown). Demyelination, neurological function, and axonal pathology were compared between a Theiler’s murine encephalomyelitis virus (TMEV)-susceptible mouse strain (SJL/J) and a MHC Class I-deficient mouse line derived from a TMEV-resistant strain (C57BL/6 × 129/J) (38). Spinal cord demyelination was extensive in both mouse strains. However, neurological disability was prominent only in SJL/J mice. Significantly, the MHC-I deficient mice exhibited relative preservation of axons while a marked axonal degeneration was observed in susceptible wild type SJL/J mice. Demyelination in the absence of axonal loss, therefore, does not necessarily cause permanent neurological disability. Thus, permanent neurological disability has been dissociated from primary demyelination, but not from axonal pathology, in rodent models. These data, however, do not exclude demyelination as an important factor in MS or EAE.

It is possible that the genetic background of SWXJ mice includes alleles that increase susceptibility to axonal transection during inflammatory demyelination. This hypothesis is supported by the data from TMEV and rat EAE studies discussed above. If most axons are transected in our mice as they are stripped of myelin, then demyelinated axons (the hallmark of MS lesions) will not be present. A complete understanding of the pathogenesis of permanent neurological disability in EAE and MS must include morphological characterization of both myelin and axons. While this is becoming the norm in MS (6, 39–41), analysis of axon pathology in EAE is just beginning to be investigated (17).

Recent studies have correlated axonal pathology (3, 17) and axonal transection (5) with inflammation in demyelinated lesions of MS brain. In addition, Kornek et al (17) quantitated axonal pathology in a rat EAE model using β-APP as a measure of acute axonal injury. As described in MS, axonal pathology correlated with the inflammatory activity of the lesions. It was concluded that acute axonal injury occurs in a short time frame after onset of inflammation and demyelination. Since the appearance of APP immunoreactivity within axons represents inhibition of fast axonal transport, axonal degeneration and loss can only be inferred. The present study extends these observations by correlating the number of relapses with axonal loss and with end-stage neurological disability in RR-EAE mice. Permanent neurological disability is more likely to occur following lesions in long motor and sensory tracts with limited compensatory pathways. In this regard, most primary progressive MS patients have a prevalence of spinal cord pathology (6, 42–44). Likewise, most lesions in our EAE mice occur in the spinal cord (13) and thus produce neurological disability. Initially, the severity of this disability is proportional to the degree of inflammation and both the inflammation and disability are reversible. However, at end-stage EAE, the extent of axonal loss determines the level of permanent neurological disability. Furthermore, the present study suggests that, with each succeeding inflammatory attack, the extent of axonal loss increases. Cumulative axon loss, in turn, would lead to an increasing level of irreversible neurological disability.

The data discussed above correlate axonal pathology and loss with permanent neurological disability in 2 animal models of MS: TMEV-induced disease and EAE. Both models provide provocative data regarding the possible pathogenic mechanisms responsible for permanent neurological disability in MS. Neither model, however, mimics the precise pathogenesis of MS. Although frequent relapses in the first 2 yr of MS predict a more rapid clinical course (45), correlation between relapse rate and permanent neurological disability is not strong (46–49). There are several potential explanations for this dissociation of inflammatory attacks, relapse rate, and disability. Inflammatory lesions in MS patients occur throughout the central nervous system and many are located in “silent”
regions that do not produce transient neurological disability. This conclusion is based upon reports that enhancing MRI lesions can outnumber neurological attacks by 10 to 1 (46).

It has been proposed that axonal degeneration starts early in MS, but that the axon loss must exceed a threshold before progressive un-remitting clinical symptoms appear (25). In end-stage EAE mice with a clinical score of 0, axonal loss was 30% and 15% in the cervical and lumbar cord, respectively, and this was significantly greater than in first attack mice with a clinical score of 4. These data support the hypothesis that a threshold of axonal loss must be surpassed before irreversible neurological disability occurs. The 45% and 60% axonal loss in chronically paralyzed mice (clinical score of 4) approaches the 68% axonal loss in spinal cord lesions of paralyzed MS patients (6). It is likely that additional mechanisms of axonal loss, such as degeneration of chronically demyelinated axons, are occurring in chronic MS patients. Axonal loss in mice with clinical score 2 was intermediate between that found in mice with scores of 0 and 4. Although previous reports have described axonal pathology in rodent models of MS (17, 38), this report is the first to correlate axonal loss with end-stage disability.

Manipulation of the immune system as done in EAE can mimic salient features of MS pathogenesis. The chronic relapsing EAE model described in this report should prove useful in unraveling inflammatory mechanisms responsible for axonal transection and permanent neurological disability. A focus on axonal pathology and early treatment has emerged in MS research (3, 6, 25, 50). Four therapies (glatiramer acetate and 3 types of interferon-β) have reduced gadolinium-enhancing lesions and possibly neurological disability in RR-MS patients (51). These partially effective treatments will require supplementation with neuroprotective or other therapies to inhibit the neurological deficits produced by axon loss. Since neurological disability and relapse rate in our model is partially ameliorated by interferon-β treatment (13), it should also be useful in testing combinatorial therapies. This is particularly relevant to MS as large-scale placebo arms will not be included in future clinical trials because most RR-MS patients will be maintained on currently approved therapies.

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REFERENCES
22. Bö L, Mörk S, Kong PA, Nyland H, Pardo CA, Trapp BD. Detection of MHC class II-antigens on macrophages and microglia, but