

AXONAL TRANSECTION IN THE LESIONS OF MULTIPLE SCLEROSIS

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ABSTRACT

Background Multiple sclerosis is an inflammatory demyelinating disease of the central nervous system and is the most common cause of neurologic disability in young adults. Despite antiinflammatory or immunosuppressive therapy, most patients have progressive neurologic deterioration that may reflect axonal loss. We conducted pathological studies of brain tissues to define the changes in axons in patients with multiple sclerosis.

Methods Brain tissue was obtained at autopsy from 11 patients with multiple sclerosis and 4 subjects without brain disease. Fourteen active multiple-sclerosis lesions, 33 chronic active lesions, and samples of normal-appearing white matter were examined for demyelination, inflammation, and axonal pathologic changes by immunohistochemistry and confocal microscopy. Axonal transection, identified by the presence of terminal axonal ovoids, was detected in all 47 lesions and quantified in 18 lesions.

Results Transected axons were a consistent feature of the lesions of multiple sclerosis, and their frequency was related to the degree of inflammation within the lesion. The number of transected axons per cubic millimeter of tissue averaged 11,236 in active lesions, 3138 at the hypocellular edges of chronic active lesions, 875 in the hypocellular centers of chronic active lesions, and less than 1 in normal-appearing white matter from the control brains.

Conclusions Transected axons are common in the lesions of multiple sclerosis, and axonal transection may be the pathologic correlate of the irreversible neurologic impairment in this disease. (N Engl J Med 1998;338:278-85.)

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MULTIPLE sclerosis is a disease of the central nervous system characterized by multicentric inflammation and destruction of myelin. The primary cause of multiple sclerosis is unknown. Environmental risk factors¹ and multiple genetic loci²⁻⁴ contribute to susceptibility to the disease. The onset of the symptoms of multiple sclerosis is often associated with breakdown of the blood-brain barrier, as visualized by magnetic resonance imaging.^{5,6} Inflammatory mediators block nerve conduction at the nodes of Ranvier,⁷ and soluble and cellular effector mechanisms destroy myelin.^{8,9} The intensity of this process, its resolution, and possibly remyelination determine the severity and duration of the clinical symptoms and recovery after each exacerbation. Acute relapses respond well to corticosteroid therapy,¹⁰ providing

further evidence that inflammation is central to the disease process.

In more than 50 percent of patients with multiple sclerosis, steadily progressive clinical deterioration develops after a 10-to-15-year relapsing-remitting disease course.¹¹ This chronic progressive stage of the disease is much less responsive to antiinflammatory-drug therapy. Although it was formerly assumed that axons were spared from the destructive process, recent studies indicate that axonal injury in multiple sclerosis may cause permanent neurologic dysfunction. Neurologic disability in patients with multiple sclerosis has been correlated with atrophy in the spinal cord,¹² cerebellum,¹³ and cerebral cortex,¹⁴ and the neuronal marker *N*-acetyl aspartate is decreased in multiple-sclerosis lesions according to magnetic resonance spectroscopy.¹⁴⁻¹⁷ In patients with relapses, reduced *N*-acetyl aspartate is restricted to the area of the lesion, whereas patients with chronic progressive multiple sclerosis have reduced *N*-acetyl aspartate even in normal-appearing white matter.¹⁸ This observation suggests the spread of axonal pathologic changes, wallerian degeneration, or both.^{19,20}

Axonal pathologic changes in multiple sclerosis were mentioned by Charcot²¹ and have been reported in an experimental model of immune-mediated demyelination.²² Few studies have rigorously characterized axonal pathologic changes in the brains of patients with multiple sclerosis. A recent study reported axonal accumulation of the amyloid precursor protein in multiple-sclerosis lesions and raised the possibility of irreversible axonal damage.²³ Using three-dimensional imaging of brain sections, we have attempted to identify the pathologic changes in axons in multiple sclerosis.

METHODS

Tissue

We studied tissue from the brains of 11 patients with multiple sclerosis (age at death, 18 to 62 years; 8 women and 3 men). Forty-seven demyelinated lesions were identified, and each was characterized as active or chronic active, as described previously.²⁴ Active lesions contained abundant and evenly distributed major-

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histocompatibility-complex (MHC) class II-positive cells throughout the lesion and myelin-protein-positive inclusions within the macrophages. In chronic active lesions, MHC class II-positive cells were abundant at the edges of lesions and less frequent in the center. On the basis of these criteria, 14 active and 33 chronic active lesions were identified.

Immunocytochemistry

The tissue was fixed in 4 percent paraformaldehyde, protected in 70 percent sucrose, placed on the stage of a sliding microtome, and frozen. Free-floating sections (30 μ m thick) were cut without exposure to solvents or other embedding mediums. Sections were rinsed in phosphate-buffered saline four times for 5 minutes each, microwaved twice for 5 minutes each in 10 mM citrate buffer (pH 6.0), incubated in 3 percent hydrogen peroxide and 10 percent Triton X-100 for 30 minutes, and immunostained by the avidin-biotin complex procedure and with diaminobenzidine, as described previously.²⁴ Sections for confocal microscopy were pretreated as described above, incubated with two primary antibodies, and then incubated with fluorescein-conjugated and Texas red-conjugated secondary antibodies (Jackson Laboratories, West Grove, Pa.), as described previously.²⁵

Antibodies

The following well-characterized primary antibodies were used: mouse anti-MHC class II and rabbit anti-myelin basic protein (Dako, Carpinteria, Calif.), mouse anti-proteolipid protein (gift from Nigel Groom), rabbit antiferritin (Sigma Immunochemicals, St. Louis), and mouse anti-nonphosphorylated neurofilament (SMI-32) (Sternberger Monoclonals, Baltimore).

Confocal Microscopy

Sections were analyzed on a Leica Aristoplan laser scanning microscope (Leitz Wetzlar, Heidelberg, Germany). Individual confocal optical sections represented 0.5- μ m axial resolution. The entire thickness of the section was scanned. The images presented here consist of 16 to 32 optical sections combined to form a "through-focus" image. Fluorescence was collected individually in the green (fluorescein) and red (Texas red) channels to eliminate "bleed-through" from either channel. The yellow is a result of stacking multiple images and not of colocalization of antigens.

Quantification of Terminal Swellings

Tissue from five brains, which included 5 active and 13 chronic active lesions, was selected for quantitative analysis of axonal transection. Sections immunostained with nonphosphorylated-neurofilament antibodies and diaminobenzidine were used for quantification. Only swellings at the terminal ends of axons, as verified by moving the plane of focus up and down through the section, were counted. To eliminate regional variations in the distribution of terminal-axon ovoids, all areas of multiple-sclerosis lesions were analyzed. The tissue volume (in cubic millimeters) was calculated by multiplying the total two-dimensional area of the grid that was analyzed by the thickness of the section (30 μ m). Terminal ovoids in multiple-sclerosis lesions, 10 microscopical fields of normal-appearing white matter in multiple-sclerosis blocks, and 20 microscopical fields of white matter from control brains were quantified. The data were compared by the Wilcoxon rank-sum test and mixed-model analysis.

RESULTS

Axonal Pathologic Changes in Multiple-Sclerosis Lesions

Axonal pathologic changes, demyelination, and inflammation were studied in serial sections of individual multiple-sclerosis lesions immunostained with antibodies to proteolipid protein, MHC class II

molecules, and nonphosphorylated neurofilament (SMI-32) (Fig. 1). Proteolipid protein is the major structural protein of central nervous system myelin,²⁶ and its absence in white matter indicates areas of demyelination. MHC class II molecules are expressed by activated monocytes and microglia concentrated in and around multiple-sclerosis lesions,²⁴ and their abundance and distribution have been used to characterize different stages of demyelinating lesions.^{23,27,28} Nonphosphorylated neurofilaments are abundant in neuronal cell bodies and dendrites.²⁹⁻³¹ Neurofilaments in healthy myelinated axons are heavily phosphorylated and not stained by SMI-32 antibodies.²⁹ SMI-32 immunoreactivity in normally myelinated regions of the brain provides a sensitive marker for demyelination or axonal pathologic changes. SMI-32 immunoreactivity was rare in normal-appearing white matter from control brains (data not shown). In contrast, all 47 multiple-sclerosis lesions analyzed contained abundant SMI-32-positive axons.

Figure 1 shows a lesion classified as active because of the absence of proteolipid-protein immunoreactivity (Panel A) and the abundance and even distribution of MHC class II-positive cells (Panel B) throughout the demyelinated area. The acuteness of the demyelination is also indicated by the detection of proteolipid-protein-positive myelin debris within the lesion (Fig. 1A, arrowheads). Abundant SMI-32 immunoreactivity was detected within the area of demyelination (Fig. 1C). This staining appeared as thin lines and small dots, which represent demyelinated axons viewed in longitudinal and transverse orientation, and as larger ovoids that may represent damaged axons.

Several patterns of SMI-32 staining were detected in and near multiple-sclerosis lesions, and these patterns varied on the basis of lesion activity. In active lesions, small-diameter axons and large ovoids (Fig. 2A) were distributed randomly throughout the demyelinated area. In chronic active lesions, SMI-32-positive axons and ovoids were concentrated at the actively demyelinating edges (Fig. 2B). Both were less abundant than those found in active lesions, and the ovoids were of smaller diameter. The centers of chronic active lesions contained fewer SMI-32-positive axons and ovoids than the borders (data not shown).

Individual axons displayed several patterns of SMI-32 immunoreactivity. Most abundant were continuous lines of SMI-32 immunoreactivity (Fig. 2A and 2B), which indicated intact demyelinated axons. Some axons contained discontinuous lines of SMI-32-positive dots (Fig. 2C). These staining patterns are consistent with axonal degeneration distal to the sites of transection. Other axons showed dramatic changes in caliber, including alternating constrictions and dilatations or single swellings that extended from a thin axon (Fig. 2D).

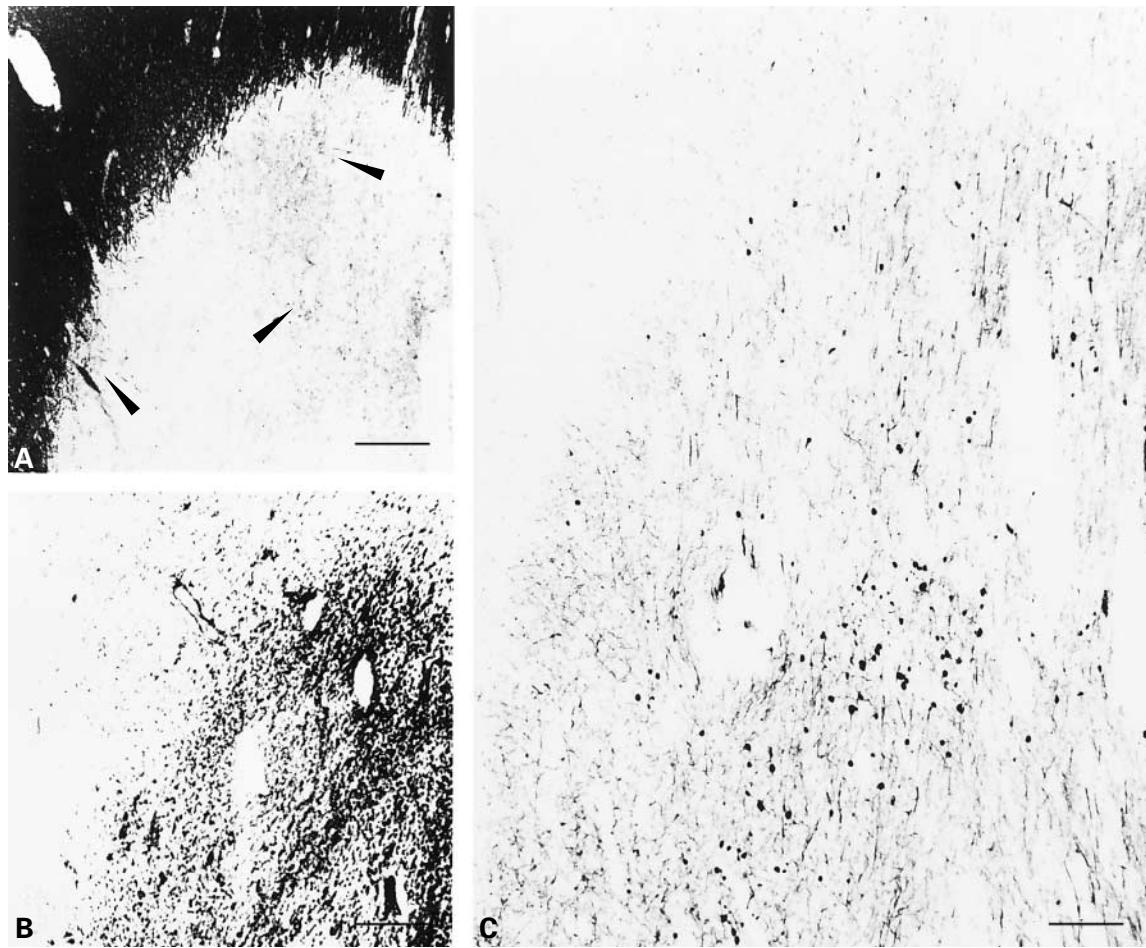


Figure 1. Serial Sections of Multiple-Sclerosis Lesions Immunostained for Myelin (Panel A), MHC Class II (Panel B), and Nonphosphorylated Neurofilaments (Panel C).

This figure shows an active multiple-sclerosis lesion characterized by loss of myelin (Panel A), myelin debris within the demyelinated area (Panel A, arrowheads), even distribution of numerous MHC class II-positive cells throughout the demyelinated area (Panel B), and abundant expression of nonphosphorylated neurofilaments within the lesion (Panel C). The scale bars in Panels A and B represent 400 μm ; the scale bar in Panel C represents 235 μm .

Axonal Transection in Multiple-Sclerosis Lesions

The most striking axonal alterations in multiple-sclerosis lesions were ovoids of intense SMI-32 staining. These structures resembled terminal axonal ovoids or end bulbs that resulted from axon transection.³²⁻³⁵ To confirm this interpretation, sections were stained with SMI-32 and fluorescence-labeled antibodies and examined by confocal microscopy, and computer-based three-dimensional reconstructions of entire ovoids were generated. Figure 3A shows an active lesion with many axonal ovoids. On the basis of three-dimensional analysis, the vast majority of ovoids retained only a single axonal connection (Fig. 3A, arrows). Axonal ovoids with dual axonal connections were rare (Fig. 3A, arrowhead). This analysis estab-

lished that the majority of SMI-32-positive axonal swellings were terminal ends of axons.

Axonal Transection in Relation to Demyelinating Activity in Multiple-Sclerosis Lesions

Inflammatory demyelination is the pathologic hallmark of multiple sclerosis. The relation between SMI-32-positive terminal ovoids and active demyelination was studied by confocal microscopy. Figure 3B shows the distribution of SMI-32 (green) and myelin basic protein (red) at the edge of a chronic active lesion. Three large-diameter axons are intensely stained by SMI-32 antibodies. The irregular and discontinuous distribution of myelin basic protein immunoreactivity around these axons indicates ongoing demyelination. The two lower, large-diameter

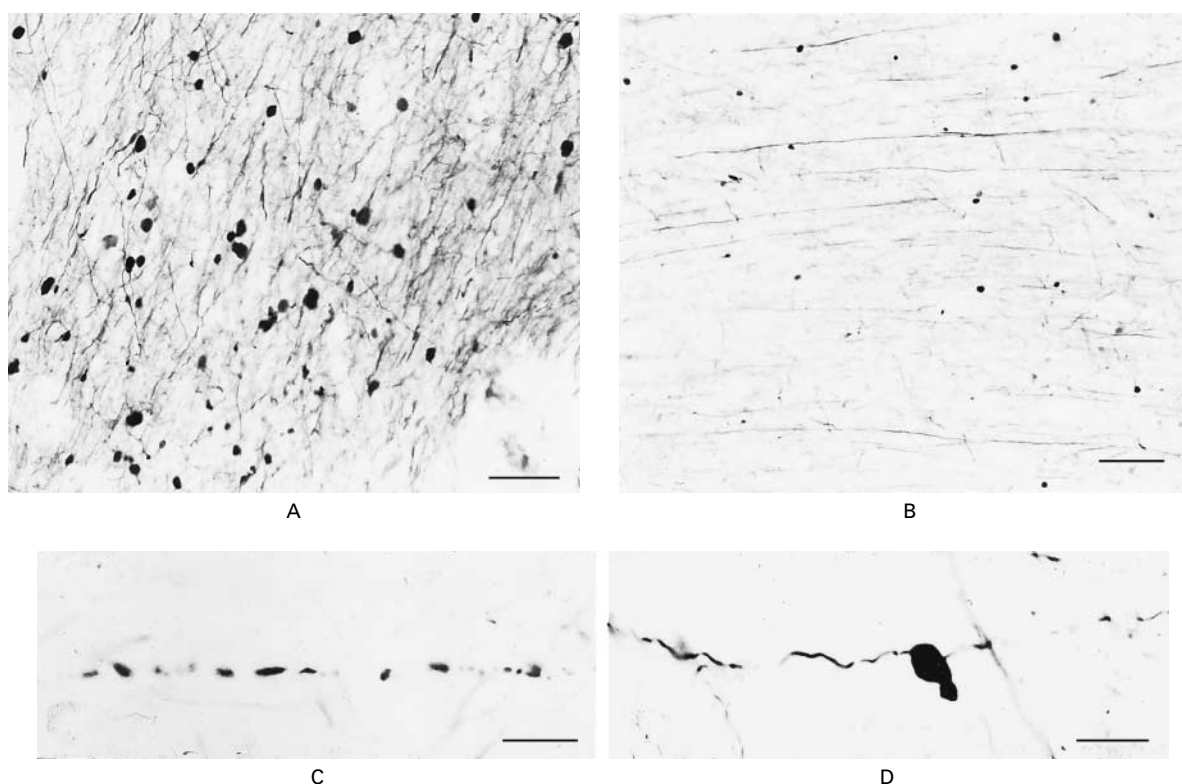


Figure 2. Patterns of Axonal Pathologic Changes in Multiple-Sclerosis Lesions.

In active lesions (Panel A), SMI-32-positive ovoids and axons are abundant. At the edges of chronic active lesions (Panel B), nonphosphorylated-neurofilament-positive axons and ovoids are less abundant and the ovoids are smaller. Most nonphosphorylated-neurofilament-positive axons in multiple-sclerosis lesions have a normal appearance (Panels A and B). Some have discontinuous staining for nonphosphorylated neurofilaments (Panel C), which is characteristic of axonal degeneration. Other axons have constrictions, dilatations, or large swellings (Panel D). The scale bars in Panels A and B represent 60 μm ; the scale bars in Panels C and D represent 52 μm .

fibers had normal-appearing axonal cylinders, whereas the upper fiber terminated in a large ovoid (Fig. 3B, arrow). Figure 3C is another confocal image of myelin basic protein (red) and SMI-32 (green) staining at the border of a lesion. Many SMI-32-positive, myelin basic protein-negative fibers extended into normal-appearing white matter, where they remained SMI-32-positive but were surrounded by normal-appearing myelin internodes (Fig. 3C, arrowheads). Occasionally, an SMI-32-positive axon that was surrounded by normal-appearing myelin terminated in a swelling (Fig. 3C, arrow). Confocal analysis of these and similar fibers provided additional evidence that most SMI-32-positive ovoids were terminal ends of axons.

Cells of monocyte origin are effectors in tissue destruction and phagocytosis in multiple-sclerosis lesions. To study the association between macrophages or microglia and terminal axonal ovoids, sections were immunostained for SMI-32 and ferritin (a marker for cells of monocyte origin) and analyzed by confocal microscopy. In the center of an active lesion

(Fig. 3D), phagocytic macrophages were abundant and were closely associated with or partially surrounded terminal axonal ovoids (Fig. 3D, arrows). Normal-appearing SMI-32-positive axons (Fig. 3D, arrowheads) were not surrounded by phagocytic macrophages. At the borders of chronic active lesions (Fig. 3E), the majority of ferritin-positive cells (red) were process-bearing microglia. SMI-32-positive axons (green) were abundant; most had a normal appearance and no consistent association with activated microglia (Fig. 3E, arrowheads). However, two terminal axonal swellings were engulfed by activated microglia (Fig. 3E, large arrows), whereas a swelling with dual axonal connections was not ensheathed by microglia or their processes (Fig. 3E, small arrow).

Quantification of Axonal Transection

The results described above established a strong qualitative correlation between axonal pathologic changes and areas of demyelination in 47 lesions from 11 patients with multiple sclerosis. To determine the quantitative relation between axonal

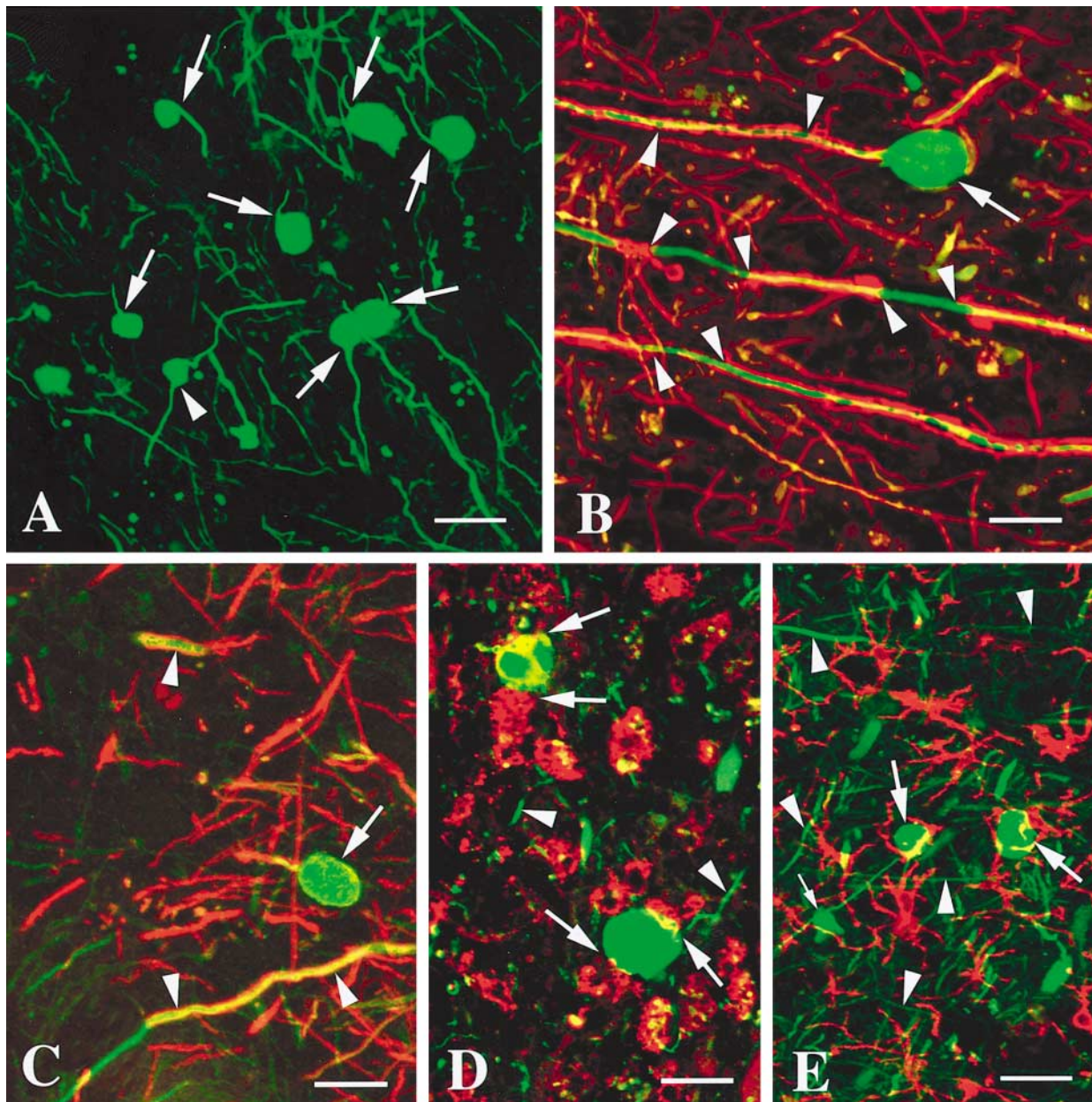


Figure 3. Confocal Microscopical Images of Axonal Changes in Multiple-Sclerosis Lesions.

Nonphosphorylated neurofilaments are green in all panels. Red indicates myelin in Panels B and C and macrophages or microglia in Panels D and E. Panels A and D show the centers of active lesions. Panels B, C, and E show the edges of active lesions. Panel A shows “stacked images” of terminal axonal ovoids with single axonal connections (arrows), an axonal ovoid with dual axonal connections (arrowhead), and many normal-appearing axons. Panel B shows three large, nonphosphorylated-neurofilament-positive axons undergoing active demyelination (arrowheads). One axon ends in a large terminal ovoid (arrow). Panel C shows some axons terminating at the ends of normal-appearing myelin internodes (arrow), and many axons that express nonphosphorylated neurofilaments surrounded by normal-appearing myelin (arrowheads). In Panels D and E, macrophages (red in Panel D) and microglia (red in Panel E) surround and engulf terminal axonal swellings (large arrows) but have no consistent association with normal-appearing axons (arrowheads) or swellings in nontransected axons (Panel E, small arrow). The scale bar in Panel A represents 64 μm ; the scale bars in Panels B, C, D, and E represent 45 μm .

transection and regions of demyelination and inflammation, we counted the number of SMI-32-positive terminal axonal swellings in 5 active and 13 chronic active lesions selected from five well-preserved brains of patients with multiple sclerosis. Transected axons within chronic active lesions were counted in the cores and at the edges of the lesions. The clinical features of the five patients are shown in Table 1. One had acute progressive disease with a course of only 2 weeks; another had primary progressive disease with a 5-year course; and three had secondary progressive disease with durations ranging from 7 to 27 years.

The average number of SMI-32-positive terminal ovoids per cubic millimeter of tissue was 17 in 11 areas of normal-appearing white matter from brains of patients with multiple sclerosis and less than 1 in 5 blocks from four control brains (Table 2). The average number of terminal axonal ovoids per cubic millimeter of tissue was 11,236 in active lesions, 3138 in the demyelinating borders of chronic active lesions, and 875 in their inactive centers. All multiple-sclerosis lesions contained significantly more terminal axonal ovoids than did normal control tissue. Active lesions contained significantly more terminal ovoids than the edges or cores of chronic active lesions, and terminal ovoids were significantly more numerous at the edges of chronic active lesions than in their cores. All lesion areas contained significantly more terminal ovoids than nonlesion areas in sections of brains of patients with multiple sclerosis. These results establish that axonal transection is a consistent and abundant feature of multiple-sclerosis lesions and that its incidence is related to the degree of inflammation.

DISCUSSION

These results establish that axonal transection is a consistent consequence of demyelination in the brains of patients with multiple sclerosis. The use of 30- μ m-thick sections and computer-based three-dimensional reconstruction identified the vast majority of axonal ovoids as terminal ends of axons. The number of terminal axonal ovoids per cubic millimeter of tissue averaged 11,236 in active lesions, 3138 at the edges of chronic active lesions, 875 at the centers of chronic active lesions, and less than 1 in white matter from control brains. These results are consistent with and extend those of a recent report describing amyloid precursor protein-positive axonal swellings in 10- μ m-thick paraffin sections of multiple-sclerosis lesions.²³ Since many multiple-sclerosis lesions have volumes of several cubic centimeters, these data establish axons as a major disease target in patients with multiple sclerosis.

The finding of extensive axonal transection in multiple-sclerosis lesions has important clinical and therapeutic implications. Historically, it has been assumed that the disease process in multiple sclerosis

TABLE 1. CLINICAL FEATURES OF PATIENTS WITH MULTIPLE SCLEROSIS WHOSE BRAIN TISSUE WAS USED FOR QUANTITATIVE STUDIES.

PATIENT No.	AGE (YR)/SEX	DISEASE DURATION	DISEASE CATEGORY
1	18/F	2 wk	Acute progressive
2	29/F	5 yr	Primary progressive
3	49/F	7 yr	Secondary progressive
4	52/M	22 yr	Secondary progressive
5	56/F	27 yr	Secondary progressive

TABLE 2. DISTRIBUTION AND NUMBER OF TRANSECTED AXONS IN MULTIPLE-SCLEROSIS LESIONS.

TISSUE (NO. OF PATIENTS)	NO. OF LESIONS ANALYZED	NO. OF TRANSECTED AXONS/mm ³ *
Active lesions (3)	5	11,236 \pm 2775
Chronic active lesions (4)	13	
Edge		3138 \pm 688
Core		875 \pm 246
Nonlesion white matter (5)	11	17 \pm 2.8
Control white matter (4)	5	0.7 \pm 0.7

*Plus-minus values are means \pm SE. $P < 0.018$ for the comparison between multiple-sclerosis samples and control white matter, by the Wilcoxon rank-sum test. For patients with multiple lesions the median value was used. $P < 0.001$ for the comparison between active lesions and the edges of chronic active lesions, $P < 0.001$ for the comparison between active lesions and the cores of chronic active lesions, and $P < 0.01$ for the comparison between the edges and the cores of chronic active lesions, by mixed-model analysis, which incorporates correlations for all lesions in a single patient.

spares axons. Major therapeutic and research efforts have been directed toward limiting immune-mediated damage to myelin and promoting remyelination. The treatment of multiple sclerosis remains unsatisfactory, with chronic disease progression being the principal clinical challenge. This report and recent results from magnetic resonance imaging studies indicate a need for noninvasive techniques that monitor axonal pathologic changes in patients with multiple sclerosis. The development of neuroprotective therapies should become an objective of multiple-sclerosis research. It will be of particular interest to define the consequences in terms of axonal injury of therapy with high-dose pulsed corticosteroids, commonly used to treat relapses,¹⁰ and interferon beta, which is indicated for patients with relapsing-remitting disease.^{36,37}

The findings in this study provide an additional rationale for early treatment with drugs that reduce

inflammation, since axonal transection is irreversible and is most abundant in areas of inflammation. However, the effects of specific antiinflammatory strategies on axonal integrity will need careful evaluation. Ultimately, neuroprotective factors may be one element of an effective, comprehensive therapeutic strategy for multiple sclerosis.

Axonal transection was abundant in active and chronic active lesions from patients with durations of clinical disease ranging from 2 weeks to 27 years. During relapsing–remitting stages of the disease, the restoration of conduction along demyelinated axons, redundant neuronal pathways, or axonal sprouting may compensate for the destruction of axons. Our results suggest that a threshold of axonal loss is eventually reached beyond which patients have progressive neurologic deterioration. If axonal pathologic changes begin at the onset of disease, as our data suggest, aggressive early treatment with neuroprotective agents should be considered.

Axonal destruction in multiple-sclerosis lesions could result from direct immunologic attack on axons, from soluble inflammatory mediators, or from secondary effects of chronic demyelination. A specific immune attack on axons seems unlikely, because most axons survive the demyelinating process and have no consistent association with cells of monocytic origin. Since the greatest degree of axonal transection occurred in areas of active demyelination and inflammation, we consider it most likely that demyelinated axons are vulnerable to inflammatory environments and that axonal transection is caused by proteolytic enzymes, cytokines, oxidative products, and free radicals produced by activated immune and glial cells.^{38–40}

The possibility that chronic demyelination may lead to axonal degeneration was also supported by the finding of terminal axonal ovoids in the centers of chronic active lesions. Myelination provides an extrinsic trophic signal to axons that increases axonal caliber,^{41,42} and the loss of this effect can result in axonal degeneration. Although the precise nature of this trophic effect is unknown, degeneration and atrophy have been described in mouse axons that are normally myelinated but are deficient in the myelin-associated glycoprotein.⁴³ As a member of the immunoglobulin gene superfamily that is localized exclusively in the adaxonal membrane of central nervous system myelin,⁴⁴ myelin-associated glycoprotein may directly or indirectly be part of a ligand system that modulates the maturation and survival of myelinated axons.

Neurofilaments are the principal constituent of the axonal cytoskeleton,⁴⁵ and their phosphorylation states are dynamically regulated by myelination, demyelination, and intrinsic axonal pathologic changes. Developmentally, myelination increases neurofil-

ament phosphorylation, which in turn increases the lateral extension of neurofilament side arms.^{46,47} This increases neurofilament spacing, axonal caliber, and axonal conduction velocity.^{42,48} In animal models, demyelination or dysmyelination causes decreased neurofilament phosphorylation, reduced axonal caliber, and an increase in nonphosphorylated neurofilament epitopes.^{49,50} Therefore, it was expected that SMI-32 immunoreactivity would be increased in multiple-sclerosis lesions and that most SMI-32–positive axons would appear normal. In addition to axonal transection, two patterns of axonal pathologic changes were identified. Discontinuous SMI-32 staining similar to that described in axonal degeneration (Fig. 2C) provided additional evidence of axonal transection in multiple-sclerosis lesions. Other axons had significant alterations in caliber (Fig. 2D) and represented damaged axons that retained active-transport systems and connection to neuronal perikarya.

Axons distant from multiple-sclerosis lesions also displayed alterations in neurofilament phosphorylation. Although most appeared normally myelinated, some ended in terminal ovoids. This finding is consistent with the results of magnetic resonance spectroscopy studies that detected reduced *N*-acetyl aspartate in regions where there were no visible lesions.²⁰ Reduced *N*-acetyl aspartate may therefore reflect potentially reversible axonal dysfunction due to demyelination,⁵¹ as well as irreversible axonal degeneration distal to sites of transection. Since myelin ensheathment governs the activity of the axonal kinases and phosphatases that regulate neurofilament phosphorylation, myelination may also modulate the activity of aspartoacylase, the enzyme that hydrolyzes *N*-acetyl aspartate to aspartate and acetate. *N*-Acetyl aspartate–derived acetate can be used to make acetyl coenzyme A.⁵² *N*-Acetyl aspartate may therefore be part of an energy-storage system in axons that helps meet the energy demands of saltatory conduction. In the absence of myelination, axonal energy demands may be reduced and *N*-acetyl aspartate levels diminished. The results of this study highlight the considerable importance of clarifying the implications of reduced *N*-acetyl aspartate in multiple-sclerosis lesions, and they stress the need to develop or improve noninvasive methods of monitoring and treating axonal pathologic changes in patients with multiple sclerosis.

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