Axonal changes in chronic demyelinated cervical spinal cord plaques

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Summary

Imaging and pathomorphological studies in multiple sclerosis suggest that axonal injury and axonal loss are playing a crucial role in those with persistent disability and long-standing disease. Although the existence of axonal injury in multiple sclerosis is proven, especially in the zone of active inflammation, the effect of chronic inflammation on the axons remains elusive. The aim of this study was to perform a quantitative morphometrical analysis, estimating axonal loss and evaluating axonal degenerative changes in cervical spinal cord samples of patients suffering from secondary progressive multiple sclerosis. Completely demyelinated plaques, normal appearing white matter (NAWM) and control material from anatomically identical regions of the cord have been compared. Neurofilament immunostaining was used for identification of the axons. We observed a significant reduction of axonal density (number of axons/mm2) in multiple sclerosis, both in the plaque and in the NAWM compared with the control cases. Axons under ~3.3 µm diameter seemed to be more affected. The intensity of the immunostaining was significantly reduced in the plaque compared with either NAWM or control. Our results on the cervical cord combined with other observations support the concept of slow axonal degeneration rather than acute damage as a cause of chronic disability in multiple sclerosis.

Keywords: axonal loss; multiple sclerosis; spinal cord; neurofilament 200 kDa immunostaining

Abbreviations: APP = amyloid precursor protein; Cr = creatine; EDSS = Expanded Disability Status Scale; LC = lateral column; MRS = magnetic resonance spectroscopy; NAA = N-acetyl aspartate; NAWM = normal appearing white matter; NF-H = neurofilament heavy chain; PC = posterior column; RR = relapsing–remitting; SMI-32 = antibody raised against neurofilament protein epitopes known to be expressed in axonal damage; SP = secondary progressive

Introduction

Multiple sclerosis is considered to be the prototype of acquired primary demyelination in the CNS. Primary demyelination refers to the pathological picture of extensive myelin loss with relative preservation of axons (Prineas and Connell, 1978; Storch and Lassmann, 1997). In the classical relapsing–remitting (RR) form of the disease, after 10–15 years more than the half of the patients develop a steadfast progressive neurological deterioration, the secondary progressive (SP) phase. In the primary progressive form slow progression is characteristic from the beginning of the disease. In contrast to the RR phase, patients suffering from the SP course respond poorly to anti-inflammatory agents.

Earlier pathological studies, pioneered by Charcot (Charcot, 1868), using the silver impregnation method have raised the possibility of axonal degeneration (Putnam, 1936; Peters, 1968; Raine and Cross, 1989). Because the impregnation technique provides unpredictable visualization of the axons, a definite pathological conclusion regarding axonal pathology was not stated by these authors.

Imaging and pathological studies of recent years have challenged the historical view of preserved axonal integrity in multiple sclerosis. Indirect and direct evidence has been collected on axonal injury. Imaging studies have provided indirect evidence for axonal loss, bringing axonal pathology in focus again (Arnold et al., 1994, 1998; Falini et al., 1998). Atrophy of the brain and the spinal cord has been described on MRI (Filippi et al., 1996, 1997; Losseff and Miller, 1998) and a strong correlation has been found between the cervical spinal cord atrophy and the disability status of the patients measured on the Expanded Disability Status Scale (EDSS) (Losseff et al., 1996; De Stefano et al., 1998).

The in vivo 1H MRS (proton magnetic resonance
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spectroscopy) analysis of the magnetic resonance spectra of metabolites with a long T2 relaxation time provided additional information on axonal pathology by analysing the chemical composition of the brain tissue (Duncan, 1996). The 1H MRS resonance intensity of N-acetyl group containing compounds in the CNS corresponds basically to N-acetyl aspartate (NAA), an amino acid which is synthesized in the mitochondria and is confined almost exclusively to neurons (Patel and Clark, 1979; Simmons et al., 1991). In practice the ratio of NAA to creatine (Cr) (NAA/Cr) is used, as it is in proportion to the neuronal compartment in the tissue and avoids the unsettled amplitude differences of the intensity signals (Matthews et al., 1991; Miller, 1991). In such studies, partially reversible signal decrease was observed during the active inflammatory phase which becomes irreversible in the chronic inactive state (Arnold, 1992; Davie et al., 1994). The reduction has been attributed to axonal loss or degeneration (McDonald, 1994; Davie et al., 1995). The reversibility is interpreted as the result of space-occupying and metabolic changes in the tissue, which decrease the relative proportion of signal intensity from neuronal elements: the presence of oedema, reactive astrocytes, inflammatory cells, mitochondrial damage and, possibly, oligodendroglia progenitors (Arnold, 1992; Urenjak et al., 1992; De Stefano et al., 1995).

Thus, an irreversible change in the NAA/Cr ratio may represent an irreversible alteration of the neuronal content in the CNS (Arnold et al., 1998). A significant decrease of the NAA/Cr ratio was detected in the areas of inflammation in RR cases but was more pronounced in SP cases, although the lesion load (total T2 intense volume of the brain) was much more pronounced in RR patients (Narayanan et al., 1997; Arnold et al., 1998). The NAA/Cr signal decrease was also observed in normal appearing white matter (NAWM), which is assumed to be the consequence of Wallerian degeneration. In NAWM the reduction of the NAA/Cr resonance intensity ratio was observed in patients with definite, irreversible neurological signs associated with either an RR or an SP course, and was found to be unchanged during the follow-up period of 30 months (De Stefano et al., 1998). The longitudinal MRS studies found a strong correlation between the decrease of the NAA/Cr ratio and scores on the EDSS scale (De Stefano et al., 1998).

Many authors equate the decrease of the NAA/Cr ratio with axonal loss (McDonald et al., 1992; McDonald, 1994), although axonal degeneration could also be responsible for such results.

Two groups (Ferguson et al., 1997; Trapp et al., 1998) carried out pathological studies which provided direct evidence of axonal damage in multiple sclerosis by demonstrating that there is axonal transection in multiple sclerosis plaques. They found that the number of transected axons accurately correlated with inflammation: the more pronounced the inflammation, the more transected axons were found. Extensive axonal damage has been described in acute plaques and in the active regions of chronic active plaques (Ferguson et al., 1997; Trapp et al., 1998). Usually the transected axons showed bulging on the proximal transected end, but sometimes strip-like axonal swelling was observed (Ferguson et al., 1997; Trapp et al., 1998). This bulging, called ‘ovoid or spheroid’ has also been described in experimental allergic encephalomyelitis (Raine and Cross, 1989). The pathological picture of ovoid enlargement and axonal swelling has already been described in earlier pathological studies in multiple sclerosis (Shintaku et al., 1988; Barnes et al., 1991) but their possible relation to acute axonal damage has not been raised. The transected ends were stained with SMI-32 [an antibody which stains non-phosphorylated neurofilament light and neurofilament heavy (NF-H) epitopes that are expressed in axonal injury (Lee et al., 1986, 1987)] and with β-amyloid precursor protein antibody (APP) which is present in injured axons (Adams et al., 1991; Gentleman et al., 1993; Sherriff et al., 1994). The feature of discontinuous SMI-32 immunostaining is thought to represent the early phase of Wallerian degeneration on the distal axonal residue (Trapp et al., 1998). The transection is possibly the consequence of the inflammatory attack on demyelinated, vulnerable axons (Trapp et al., 1998). As it is not clear which proportion of SMI-32 or APP positive axons are irreversibly damaged, the extent of axonal loss in chronic lesions, when irreversible neurological impairment occurs, remained to be clarified.

The aim of our study was to determine quantitatively the axonal changes, i.e. axonal loss and degeneration in (i) chronic inactive completely demyelinated plaques, (ii) NAWM of SP cases, and (iii) control samples from cases without CNS disease. The cervical spinal cord has been chosen for the study for two reasons: (i) definitive positive correlation was described on MRI between cord atrophy and the EDSS (Losseff et al., 1996, 1997) and (ii) the unique anatomic organization of the cord offers the possibility to compare analogous areas both structurally and functionally. This is because the anatomical tracts in the demyelinated area can be compared with the contralateral functionally identical region. Selected spinal cord samples from clinically definite multiple sclerosis patients with at least 9 years of documented SP disease were used in the study. Only those samples were chosen in which one side contained a completely demyelinated plaque while NAWM was found in the contralateral corresponding area. Instead of the impregnation method, which only allows the ‘chance’ visualization of axons, we used the easily reproducible, reliable 200 kDa neurofilament immunostaining to detect all axons in the tissue. This antibody identifies both phosphorylated and unphosphorylated epitopes (Fig. 1).

Material and methods

Human tissue

Cervical spinal cord samples from 10 multiple sclerosis cases and from 11 control cases were used in the study. The multiple sclerosis tissue blocks were obtained from the
specimen bank of the Department of Neuropathology, National Institute of Psychiatry and Neurology, Budapest, and from the Department of Neurology, Markusovszky Hospital, Szombathely. Only samples from patients having a documented SP of at least 9 years duration were included (four men and six women, disease duration 9–21 years, age at death 32–56 years; see Table 1). The age-matched control samples were obtained from persons who died without CNS disorder diagnosed on autopsy. The control material was supplied by the Department of Pathology, ‘Jahn Ferenc’ Teaching Hospital, Budapest, and the Department of Forensic Medicine, Semmelweis University Medical School, Budapest. The control samples were dissected from identical spinal cord segments to the multiple sclerosis samples. In one case (multiple sclerosis patient aged 51 years) two control samples (one male and one female) were used. The dissected tissue blocks were fixed in 4% formalin (from 12 weeks to 2 years) and embedded in paraffin wax.

**Antibody**

Immunostaining was used in order to obtain reproducible and reliable detection of axonal neurofilaments (NF-H, or NF 200 kDa). NF-H is known to be expressed exclusively by neurons in the CNS. The structure of the protein is modified by phosphorylation (see further details in Discussion). The well-characterized mouse monoclonal anti-neurofilament 200 kDa antibody was used for the immunostaining (Novocastra Laboratories Ltd, Newcastle, UK, code: NCL-NF200-N52). The antibody reacts with both phosphorylated and unphosphorylated NF-H.

**Selection of multiple sclerosis lesions**

Lesion activity was determined by the presence of foamy macrophages and perivascular lymphocytes. Only chronic lesions with complete demyelination were examined where only a few or no macrophages or lymphocytes were seen in the centre or the periphery of the lesions (Prineas and McDonald, 1997).

Using luxol fast blue staining in combination with neurofilament immunostaining (Yao et al., 1994), only samples containing a completely demyelinated plaque on one side and NAWM on the other were selected. Spinal cord samples with plaques from C2–T1 segments were investigated (see Table 1).
Table 1 Clinical information of multiple sclerosis and control samples and the distribution of spinal cord plaques examined

<table>
<thead>
<tr>
<th>Multiple sclerosis samples</th>
<th>Control samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Cord level</td>
</tr>
<tr>
<td>44</td>
<td>C2</td>
</tr>
<tr>
<td>47</td>
<td>C2</td>
</tr>
<tr>
<td>49</td>
<td>C2</td>
</tr>
<tr>
<td>50</td>
<td>C5</td>
</tr>
<tr>
<td>51</td>
<td>C6</td>
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<td>52</td>
<td>C5</td>
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<td>52</td>
<td>C2</td>
</tr>
<tr>
<td>55</td>
<td>C5</td>
</tr>
<tr>
<td>56</td>
<td>C2</td>
</tr>
<tr>
<td>61</td>
<td>C5</td>
</tr>
</tbody>
</table>

Anti-200 kDa neurofilament immunohistochemistry

Serial sections 7 µm thick were cut on a microtome and picked up in gelatinized slides. The sections were dewaxed and rinsed in PBS (phosphate buffered saline) before immunostaining using the ABC (avidin–biotin–peroxidase complex) method. To block endogenous peroxidase activity and non-specific immunostaining, sections were immersed in PBS containing 3% H2O2 (hydrogen peroxide) before treating them with PBS containing 20% normal horse serum for 60 min at room temperature. The sections were microwaved in 0.1 M citrate buffer (pH 6) bringing them to boiling point, then cooling them for 20 min. The sections were then incubated overnight at 4°C with the primary antibody (1 : 600, raised in horse). The ABC reagent in Vectastain Elite ABC kits (Vector Laboratories Inc., Burlingame, Calif., USA) was used according to the manufacturer’s instructions. DAB (diaminobenzidine) solution, 0.05%, containing 0.01% H2O2 was prepared just before use with millipore-filtered distilled H2O. Five to six minutes of immersion was sufficient to achieve good detection of the peroxidase reaction. The sections were dehydrated and coverslipped by DePex (Fluca Chemie AG, Buchs, Switzerland) mounting medium. All sections were processed as above in parallel.

Computer aided image analysis

Based on the luxol fast blue stained serial sections, the border of each completely demyelinated plaque area was determined on the NF-H immunostained sections. In parallel, exactly the same neuroanatomical region was determined on the contralateral NAWM side of the same multiple sclerosis section, as well as on the control sample. In this way the regions of interest for the image analysis were specified. The regions of interest represent anatomically identical areas of the sections intended for detailed further investigation (i) in the plaque, (ii) in the contralateral NAWM, and (iii) in the control samples. Under ×60 magnification the whole area was examined systematically in such a way that every microscopic field was digitalized directly from the camera, which finally allowed the reconstruction of the area as a large image file by computer (Power Macintosh 7100/66av computer). This large image file was built up from 30–90 microscopic fields depending on the extent of the lesion. The identical ‘scanning’ procedure was performed on the NAWM and the control areas. We chose to study the whole lesion, not just the microscopic field samples, to avoid inter-observer differences in sample-taking. In each case the large image files were further analysed using image analysis software, NIH Image 1.61 (developed at the US National Institute of Health, Bethesda, Md., USA, and available on the Internet at http://rsb.info.nih.gov/nih-image). Twelve plaques were located in the lateral column (LC) and 10 in the posterior column (PC).

The larger and smaller diameter of the axons, the area of the axonal cross-sections and the relative intensity of the immunostaining were determined. The relative intensity of the immunostaining was acquired using a grey-scale standard during digitalization. The density scale ranged from 0 to 255 units.

Statistical analysis

The data obtained were further analysed using Excel 7.0 for Windows 95 and Statistica 5.0 software. The shrinkage caused by fixation and embedding has been calculated and values for the axonal diameter and area are given as in the living state (Palkovits et al., 1971). For the analysis the term ‘axonal density’ was determined as the number of axons/mm².

A Mann–Whitney U test was used to compare the difference in axonal density. Histograms were prepared (with a bin range of 0.2 µm) to plot the changes in axonal diameter against relative frequency (axons in a bin range/microscopic field). If the relative frequency was to be determined as the number of axons in a bin/total number of axons, then the histograms would not show the actual difference in axonal
number, but simply present the type of the distribution. The change in axonal diameter was analysed by a Kolmogorov–Smirnov test, determining the difference of the distributions in the three chosen areas. The difference in relative intensity of the NF-H immunoreaction was evaluated by a Kolmogorov–Smirnov two-sample test.

Results

Changes in the axonal density

The axonal density was determined as the number of axons/mm². It has been measured in the plaques, the NAWM and in the controls. The plaques in the LC and the PC have been considered separately. A Mann–Whitney U test showed a significant decrease of axonal density in the plaque versus control and in the NAWM versus control, both in the LC and the PC. Within the multiple sclerosis samples no significant difference was observed between the plaque and the NAWM (P < 0.16), although the density in the plaque was slightly lower (Fig. 2). It must be emphasized that the axonal density was not corrected by the extent of cord atrophy reported on MRI (Losseff et al., 1996; Losseff and Miller, 1998).

Changes in the axonal diameter

To follow the changes in axonal calibre, histograms were determined in each group. The calibre distribution proved to be a partially skewed distribution. The occurrence of axons larger than 3.3 μm diameter in the LC and 3.5 μm in the PC was not significantly different in the multiple sclerosis and control cases. The number of axons under these diameters was significantly decreased in multiple sclerosis samples. A Kolmogorov–Smirnov test found a significant difference in plaque versus control, NAWM versus control and plaque versus NAWM comparisons, P < 0.001 in all cases. In NAWM the axons over 2 μm diameter in the LCs and 1.1 μm diameter in the PCs were more preserved (Fig. 3).

Intensity of immunostaining

For semiquantitative comparison of the relative intensity of the immunostaining, all of the slides were simultaneously prepared. During digitalization the intensity values were normalized by a standard density scale. The intensity of the DAB reaction was determined using a standard density scale (units ranged from 0 to 255): plaque density, median = 141 ± 0.25 units; NAWM density, median = 166 ± 0.34 units. For the detection of the difference of the distributions a Kolmogorov–Smirnov two-sample test was used. A significant decrease of intensity could be observed on comparing the plaque with the NAWM (P < 0.001) (Fig. 4), but not between NAWM and the control (P < 0.5) (not shown in Fig. 4).

Estimation of axonal loss in chronic demyelinated multiple sclerosis spinal cord

The exact estimation of axonal loss is not possible because of a number of uncontrollable parameters: (i) no serial cervical spinal cord MRI examinations to estimate the degree of atrophy were performed in any of the cases studied; (ii) large numbers of axons enter and leave the cord in each segment from the cervical enlargement of the cord (Barson and Sands, 1977), altering the total number of axons even section by section; (iii) the plaques in the samples were located in different segments; (iv) it is not known whether the atrophy described on MRI involves the cord symmetrically. Assuming that the cord atrophy is symmetric, that the grey matter of the cord occupies equal areas in the segments of multiple sclerosis and control cases, and, from the MRI studies, about 15% atrophy (Losseff et al., 1996; Losseff and Miller, 1998), we estimate that ~65% of axons are lost in the studied SP samples.

Discussion

We determined the changes in axonal pathology and undertook a quantitative morphological analysis in (i) completely demyelinated multiple sclerosis plaques, and (ii) NAWM of SP samples, and compared these with control material from anatomically identical cervical spinal cord regions, using 200 kDa neurofilament immunohistochemistry. A significant reduction in axonal density (number of axons/mm²) was observed in the plaques and the NAWM versus the control cases. Because we used no correction factor related to the described cord atrophy on MRI (Losseff et al., 1996; Losseff and Miller, 1998), the in vivo density difference is likely to have been even more prominent. There was no significant difference in axonal density in the multiple sclerosis plaque versus the contralateral NAWM. Our findings are in accordance with the results of brain MRS observations on decreased neuronal tissue content in NAWM in the
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Fig. 2 The axonal density (number of axons/mm²) is significantly lower within the multiple sclerosis sample (either in the plaque or the NAWM) compared with control. This result confirms the existence of axonal loss in SP multiple sclerosis on a morphometric basis. The figures above were not corrected for the extent of spinal cord atrophy described on MRI. This means that even more significant differences may be present in vivo regarding the absolute quantity of axonal loss. The axonal density within the multiple sclerosis sample, the density difference between the plaque and the NAWM, was not significant.

Table 3 Axonal calibre and the skewness of calibre distribution

<table>
<thead>
<tr>
<th>Area</th>
<th>Plaque</th>
<th></th>
<th>NAWM</th>
<th></th>
<th>Control</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (µm)</td>
<td>SD</td>
<td>Skewness</td>
<td>Median (µm)</td>
<td>SD</td>
<td>Skewness</td>
</tr>
<tr>
<td>LC</td>
<td>1.38</td>
<td>1.04</td>
<td>1.86</td>
<td>1.44</td>
<td>0.93</td>
<td>1.59</td>
</tr>
<tr>
<td>PC</td>
<td>1.15</td>
<td>0.7</td>
<td>1.62</td>
<td>1.56</td>
<td>1.11</td>
<td>13.77</td>
</tr>
</tbody>
</table>

cerebral hemispheres (Husted et al., 1994; Arnold et al., 1998; Fu et al., 1998), but the greater magnitude of the changes in the spinal cord requires an explanation. Three points should be considered. (i) Almost all of the long ascending and descending neural tracts connecting the brain and the spinal cord are localized in the cervical spinal cord. The lesional involvement of these tracts is possible all along their course. We suggest that the equally decreased axonal density in the NAWM and in the plaque could present the final outcome of multisegmental long-standing inflammation affecting the tracts. In this sense, the studied lesion represents just a part of the inflammatory process. The axonal damage due to the examined lesion did not produce significant differences in axonal density within the multiple sclerosis sample, although the density was slightly lower in the plaque. (ii) The number of cases studied (10) could be considered as being a small sample in a statistical sense. It was sufficient, however, to detect the pronounced axon loss in the multiple sclerosis versus control, although the number may not be large enough to detect a genuine difference in the plaque versus NAWM. (iii) It should also be noted that the axonal density does not represent the absolute axonal number. The possible asymmetry of the cord atrophy in the plaque may mask the difference in axonal density of the samples. The examination of axonal calibre change revealed that the smaller axons tended to be more affected in the studied SP course; similar findings were presented by Ganter and colleagues (Ganter et al., 1998). We estimate from our observations an axonal loss of around 65%, but the possible confounding factors mentioned in the Results need to be kept in mind.

The intensity of NF-H immunostaining was reduced in the demyelinated area, underlying the importance of cytосkeletal
Fig. 3 The graph represents the axonal diameter profile changes in the LC of the cervical spinal cord in multiple sclerosis and control cases on a histogram. A large decrease in the number of axons of less than 2.2 \( \mu \)m diameter in the plaque and the NAWM can be observed compared with the control. The small number of axons over 3–3.3 \( \mu \)m diameter is preserved. Axons were thinner in the plaque than in the NAWM. The given diameter values are corrected regarding the shrinkage effect due to paraffin embedding.

Fig. 4 The intensity histograms of the relative density (grey value) of the immunostaining in the plaque and in the NAWM. The significantly lighter immunostaining in the plaque could relate to axonal cytoskeletal degeneration due to myelin loss.

structure disorganization in multiple sclerosis. It is known that the neurofilament network provides an obligatory and major part of the matured neuronal/axonal cytoskeleton (Nixon, 1993; Lee and Cleveland, 1996). The neurofilament network is built up from two neurofilament pools. On the one hand, the stationary pool forms the ‘skeleton’ of the axons. It consists of phosphorylated neurofilament subunits (Nixon, 1993). The so-called moving (or transport) pool, on the other hand, contains newly synthesized non-phosphorylated neurofilament units (Dahl, 1983; Nixon,
1993) being transported by slow axonal transport. The myelin ensheathment on the surface of the axolemma is known to induce phosphorylation (Dahl et al., 1989) of the C-terminal tail of the neurofilament molecules (Nixon et al., 1991; Nixon, 1993) through modification of a kinase-phosphatase balance (Lee and Cleveland, 1996) which results in the incorporation of unphosphorylated neurofilament units of the moving pool into the stationary pool (Nixon et al., 1982; Nixon, 1993). This provides the necessary axonal diameter, which in turn determines the conduction velocity of the axons (Dahl et al., 1989; Lee and Cleveland, 1996). The myelin loss mobilizes the stationary pool to the more easily degradable moving pool (Lee and Cleveland, 1996). This results in a reduction of axonal diameter, as has been observed in in vitro models (de Waegh et al., 1992), in dysmyelinating mutants (Barron et al., 1987) and physiologically at the nodes of Ranvier (Mata et al., 1992).

Demyelination in multiple sclerosis also results in a disorganization of ion channels (Moll and Ulrich, 1989) causing conduction block (Hess et al., 1986; Ingram et al., 1988). Functional recovery is aided by the increased number of sodium channels expressed in the axolemma (England et al., 1990; Vábnick et al., 1997) in partially demyelinated multiple sclerosis plaques (Moll et al., 1991), although this last study reports that, in complete demyelination, ion channel proteins disappear from the plaque.

Recent studies of axonal pathology in multiple sclerosis presume that chronic neurological impairment represents solely the clinical correlate of axonal loss (McDonald et al., 1992; McDonald, 1994). This conclusion, however, is not necessarily true (Scolding and Franklin, 1998). Basically, the presence of a neurological sign is considered as a consequence of conduction block or axonal degeneration/loss. Reversible conduction block in multiple sclerosis can be responsible for generating clinical phenomena such as remission, thermosensitive neurological signs (Uthoff’s phenomenon) or changes in visual evoked potentials. In our view the degeneration of axonal cytoskeleton in multiple sclerosis and the conduction block probably present two associated aspects of the same pathological process. Although recent studies on axonal pathology presume that chronic neurological impairment is the exclusive result of axonal loss, irreversible conduction block of degenerated axons should not be ignored. In our opinion the axonal loss could relate to the accumulated effect of long-standing inflammation and the chronic conduction block probably relates to demyelination and axonal cytoskeletal deficit.

Axonal transection is a feature coupled to the effects of inflammation (Ferguson et al., 1997; Trapp et al., 1998). The activity of the inflammation is known to correlate with T2 intensity on MRI images, the so-called lesion load (Narayanan et al., 1997). The T2 lesion load is reported to be much higher in RR multiple sclerosis than in SP multiple sclerosis (Revesz et al., 1994; Arnold et al., 1998). The estimated time of axonal degeneration after axonal transection can be measured in days, and axonal transection is a feature of ongoing inflammation. In contrast to this, the NAA signal reduction was reported to be more pronounced in SP multiple sclerosis and late RR multiple sclerosis than in early RR multiple sclerosis (Arnold et al., 1998); many years may elapse before the RR phase changes to SP. A reduction in the neuronal component was observed in regions without any inflammatory sign in the NAWM in long-term MRS studies (Arnold et al., 1994; Husted et al., 1994; Fu et al., 1998). In SP multiple sclerosis the signal reduction of MRS was found to be continuous (De Stefano et al., 1998; Fu et al., 1998).

The episodic axonal damage, in which a part of the axonal tree is lost (Ferguson et al., 1997), and the chronic conduction block (degenerated cytoskeleton, loss of Na+ channels, chronic electric silence) result in a partial neuronal functional inactivity. Destruction of part of the axonal arborization and the severe impairment in the cytoskeleton (axonal transport) could severely reduce the metabolic activity and survival mechanisms such as the growth factor uptake. These processes (low levels of growth factor concentration and electrical inactivity) could lead to cellular death in vitro. Normally, in vivo neuronal plasticity ensures that the neighbouring neurons take over the function of the lost cell. In multiple sclerosis, through damage to the axons of specific anatomical tracts that pass through the inflammatory zone, even the neighbouring cellular elements could be involved resulting in limited plasticity. By exceeding an axonal damage threshold, these processes could theoretically allow the existence of a slow but continuous neuronal loss, initiating a process of slow neuronal network breakdown. Our data do not prove this but nevertheless suggest a form of axonal-neuronal damage in multiple sclerosis other than acute axonal damage.

In conclusion, the present data are consistent with other studies which have proposed that the reduction of inflammatory activity and protection of axons, for example, by inducing remyelination, could limit axonal degeneration/loss. This may offer a therapeutic opportunity to slow the development of irreversible neurological impairment in multiple sclerosis.

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