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Acute axonal injury in multiple sclerosis
Correlation with demyelination and inflammation

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Summary
Damage to axons is taken as a key factor of disability in multiple sclerosis, but its pathogenesis is largely unknown. Axonal injury is believed to occur as a consequence of demyelination and was recently shown to be a feature even of the early disease stages. The present study was aimed at characterizing the association of axonal injury and histopathological hallmarks of multiple sclerosis such as demyelination, cellular infiltration and expression of inflammatory mediators. Therefore, axon reduction and signs of acute axonal damage were quantified in early lesion development of chronic multiple sclerosis and correlated with demyelinating activity and inflammation. Patients with secondary progressive multiple sclerosis revealed the most pronounced axonal injury, whereas primary progressive multiple sclerosis patients surprisingly showed relatively little acute axonal injury. Acute axonal damage, as defined by the accumulation of amyloid precursor protein (APP), was found to occur not only in active demyelinating but also in remyelinating and inactive demyelinated lesions with a large inter-individual variability. Only few remyelinating lesions were adjacent to areas of active demyelination. In this minority of lesions, axonal damage may have originated from the neighbourhood. APP expression in damaged axons correlated with the number of macrophages and CD8-positive T lymphocytes within the lesions, but not with the expression of tumour necrosis factor-alpha (TNF-α) or inducible nitric oxide synthase (iNOS). Axonal injury is therefore, at least in part, independent of demyelinating activity, and its pathogenesis may be different from demyelination. This has major implications for therapeutic strategies, which aim at preventing both demyelination and axonal loss.

Keywords: multiple sclerosis; axons; demyelination; inflammation

Abbreviations: APP = amyloid precursor protein; DM = inactive demyelinated lesions; EA = early active demyelinating lesions; ERM = early remyelinating lesions; iNOS = inducible nitric oxide synthase; LA = late active demyelinating lesions; LRM = late remyelinating lesions; MBP = myelin basic protein; MOG = myelin oligodendrocyte glycoprotein; NAA = *N*-acetylaspartate; PLP = proteolipid protein; TNF-α = tumour necrosis factor-alpha; WM = periplaque white matter

Introduction
Multiple sclerosis is an inflammatory disease of the CNS that causes demyelinated plaques with glial scar formation (Lassmann, 1998). Although the hallmark of the process is demyelination, destruction of axons has also been observed. There is evidence that the extent of axonal loss correlates with the reduction of *N*-acetylaspartate in quantitative magnetic resonance spectroscopy, as well as with T_1-weighted hypointensity in MRI and with the extent of CNS atrophy, for example in the spinal cord (Barnes *et al.*, 1991; Losseff *et al.*, 1996; Brück *et al.*, 1997; van Walderveen *et al.*, 1998). All these parameters correlate with clinical disability (Davie *et al.*, 1995; Losseff *et al.*, 1996; Truyen *et al.*, 1996; De Stefano *et al.*, 1997, 1998). This has led to the concept that irreversible axonal damage is an essential cause of non-remitting clinical disability and disease progression. So far, no therapy has been proven to stop or prevent axonal damage, the pathogenesis of which is largely unknown. Axonal damage has already been described in the very early literature of multiple sclerosis pathology (Charcot, 1868). Most studies on axonal injury, however, have been performed on autopsy tissue of patients, who died either from fulminant multiple sclerosis or after many years of a chronic disease course. Based on the analysis of this limited material, axon reduction was thought to occur early in the course of the disease and to correlate with the extent of inflammation (Ferguson *et al.*, 1997; Trapp *et al.*, 1998). So far, there has been little information available on the early course of chronic multiple sclerosis.

Axonal injury leads to the transection of axons and to the formation of axon spheroids at their proximal ends (Trapp *et al.*, 1998). Although there is some evidence from magnetic resonance spectroscopy that axonal reduction also appears in the normal white matter (Davie *et al.*, 1997; van Walderveen *et al.*, 1998), most axons that undergo transection are demyelinated and located within multiple sclerosis lesions. However, it is still unknown whether the same pathogenetic mechanisms cause both demyelination and axonal injury. Additionally, it is a matter of debate whether demyelination is an essential or sufficient precondition for axon damage or whether both conditions appear independently from each other. The answers to these questions are of great interest in connection with therapies that so far have focused mainly on the prevention of inflammation and demyelination (Hohlfeld, 1997).

The current study was designed to investigate whether acute axonal injury is linked to active demyelination in multiple sclerosis lesions. Therefore, brain biopsy specimens from multiple sclerosis patients were investigated histopathologically with respect to myelin and axonal damages. In addition, the extent of axonal injury was correlated with the inflammatory infiltrate and the expression of mRNA specific for two potential mediators of demyelination, tumour necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS).

Our results show acute axonal injury as revealed by the expression of the amyloid precursor protein (APP) to appear in lesions of any demyelinating activity, even during remyelination. Axonal damage increased with the number of infiltrating macrophages and CD8-positive T lymphocytes, but not with the expression of TNF-α or iNOS mRNA. Therefore, axonal injury in multiple sclerosis lesions appears, in part, to be independent of demyelinating activity, and different pathogenic mechanisms seem to be involved.

Material and methods

Patients

Brain tissue was derived from 45 CT-guided stereotactic needle or open biopsies from 42 patients. Three patients underwent two biopsies from different CNS sites at consecutive time points during the progression of their disease. The biopsies had been collected during the last 10 years. Biopsies were performed for diagnostic reasons to exclude neoplastic or infectious diseases (i.e. toxoplasmosis) after an informed consent had been obtained from each patient. Clinical information from the years before and after the biopsy was available in most cases (Table 1). In at least 16 patients, the symptoms at biopsy were the first of the disease (unknown in 11 patients). At the time of the biopsy, none of the patients fulfilled the accepted criteria for clinically definite multiple sclerosis (Poser *et al.*, 1983). In the further disease course after the biopsy, however, most patients acquired these diagnostic criteria on the basis of the clinical course and cerebrospinal fluid analysis (Table 1). In patients with a minor diagnostic certainty, the follow-up period after biopsy was short.

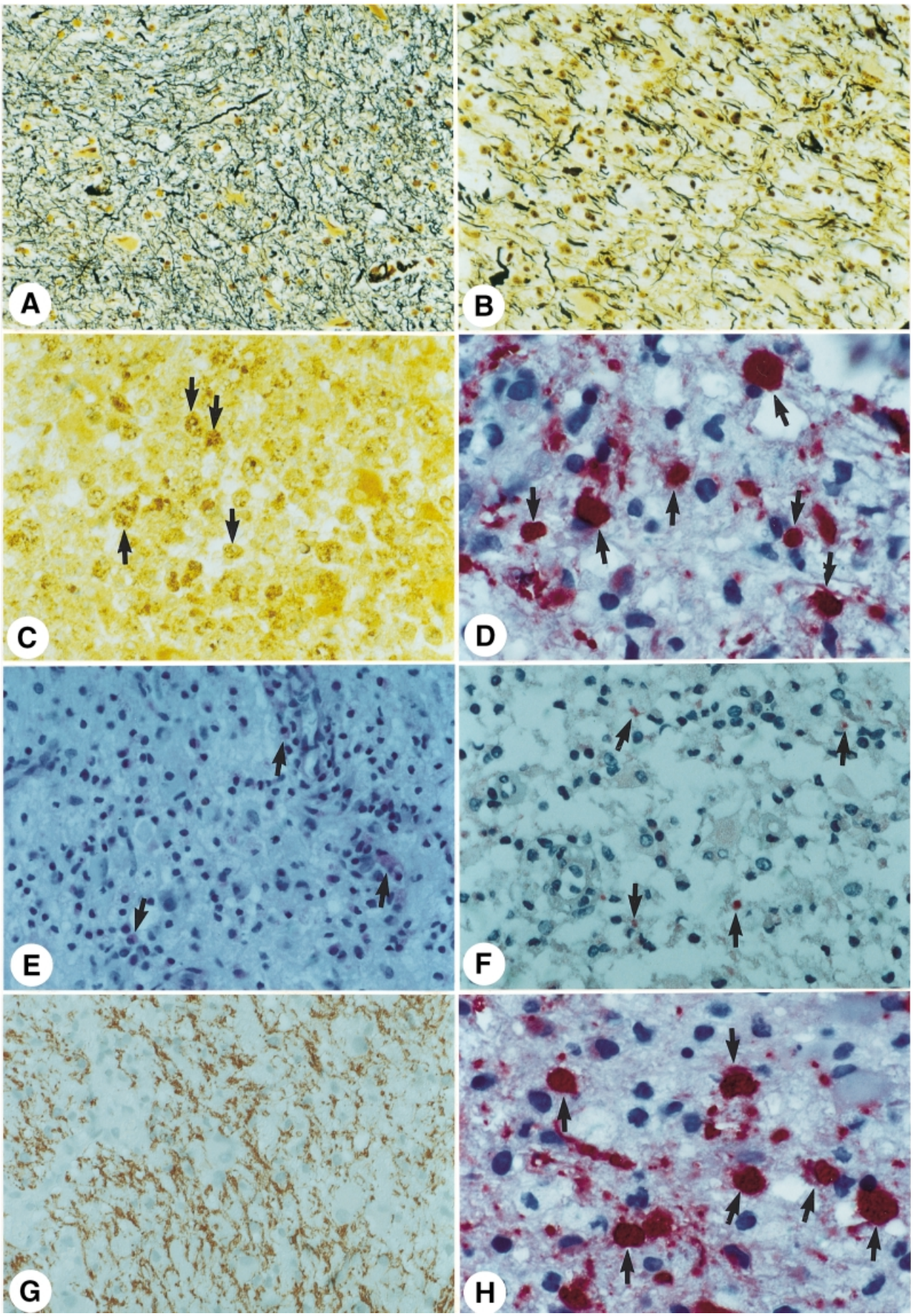
Table 1 *Patient's clinical characteristics*

Female $n = 28$, male $n = 11$, unknown $n = 3$	
Age 33 years (median), 11–64 years (range), unknown $n = 5$	
Application of diagnostic criteria during the further disease course years after biopsy* (Poser *et al.*, 1983):	
Laboratory-supported clinically definite	$n = 17$
Clinically definite	$n = 5$
Clinically probable	$n = 2$
Clinically possible	$n = 12$
Unknown	$n = 6$
Clinical course	
Monophasic (so far)	$n = 14$
Relapsing–remitting	$n = 14$
Secondary progressive	$n = 4$
Primary progressive	$n = 4$
Fulminant (Marburg's disease)	$n = 1$
Unknown	$n = 5$
Interval from first symptoms to biopsy: 90 days (median), 37 days–14 years (range)	
Immunosuppressive treatment before biopsy:	
None	$n = 18$
Prednisolone	$n = 13$
Intravenous immunoglobulin	$n = 1$
Interferon-β 1b	$n = 1$
Unknown	$n = 9$

*No patient was classified 'definite' at the time of biopsy.

Histopathology

Biopsies were performed in different centres all over Germany and sent to Göttingen's neuropathology department after completion of routine analyses. Specimens were fixed immediately with formalin and embedded in paraffin. Slices of 10 μm thickness were cut and placed on glass slides. Neuropathological staining comprised haematoxylin and eosin (HE), Luxol fast blue (LFB), periodic acid–Schiff (PAS) and Bielschowsky's silver impregnation. Immunocytochemical staining was performed with a biotin–avidin or an alkaline phosphatase–anti-alkaline phosphatase technique. The primary antibodies were anti-myelin basic protein (MBP, Boehringer Mannheim, Mannheim, Germany), anti-proteolipid protein (PLP, Dr Piddlesden, University of Cardiff, UK), anti-myelin oligodendrocyte glycoprotein (MOG, Dr Piddlesden), anti-KiMlP (macrophages, Dr Radzun, University of Göttingen, Germany), anti-27E10 and anti-MRP14 (activated macrophages, BMA Biomedicals, Augst, Switzerland), anti-CD3 (T cells, Dako, Glostrup, Denmark), anti-CD8 (Dako, Denmark) and anti-APP (Boehringer Mannheim, Germany). *In situ* hybridization followed a recently described procedure applying digoxigenin-labelled oligonucleotide probes specific for TNF-α and iNOS, respectively. Probes were generated by polymerase chain reaction (PCR), as described earlier (Bitsch *et al.*, 1998). Primers TNFα-1 (GCCAATGGCGTGGA-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GCTG) and TNFα-2 (TCGGCAAAGTCGAGATAGTCG) amplified a 390 bp fragment on exon 4 of the TNF-α gene (Nedwin *et al.*, 1985). iNOS primers were complementary to exon 12 (iNOS-1: CCACTCGGCTGCAGAATCCT) and exon 13 (iNOS-2: TGACCAAGACTTTCAATGGAATC), respectively, and amplified a 251 bp fragment (Charles *et al.*, 1993; Xu *et al.*, 1996). A second amplification of these fragments with modified primers resulted in their extension by specific RNA polymerase promoters, which allowed the synthesis of digoxigenin-labelled antisense and sense cRNA probes by *in vitro* transcription with T7 and T3 polymerases, respectively (Logel *et al.*, 1992). Non-radioactive *in situ* hybridization adhered exactly to a published protocol (Bitsch *et al.*, 1997, 1998).

Classification of multiple sclerosis lesions

All lesions fulfilled the generally accepted criteria for the diagnosis of multiple sclerosis (Allen, 1991). There was confluent demyelination within the white matter with an inflammatory infiltrate consisting of lymphocytes, macrophages and microglia. No haemorrhages were present within the lesions (excluding haemorrhagic encephalomyelitis). Inflammation and demyelination were not restricted to the perivascular areas (excluding acute demyelinating encephalomyelitis). Lesions were classified with respect to their demyelinating activity as recently described (Brück *et al.*, 1995). Early active demyelinating lesions (EA, n = 24) were located at the plaque border, partially demyelinated and diffusely infiltrated by macrophages/microglia that contained myelin proteins (MOG and MBP). In late active demyelinating lesions (LA, n = 13), demyelination was more advanced and infiltrating macrophages/microglia contained MBP but not MOG, which has a shorter half-life during myelin degradation. Inactive demyelinated lesions (DM, n = 20) were also infiltrated by macrophages/microglia, but these cells contained only PAS-positive myelin degradation products and no myelin proteins that could be detected by immunocytochemistry. Demyelination was complete. In early remyelinating lesions (ERM, n = 28), thin myelin sheaths indicated remyelination. There was pronounced infiltration by macrophages/microglia and lymphocytes. Late remyelinating lesions (LRM, n = 12) showed only minor inflammation but marked astrocyte proliferation with gliosis. The periplaque white matter (WM, n = 38) appeared normal by means of light microscopy and revealed no signs of myelin degradation or phagocytosis.

Morphometry

Relative axonal density was determined in sections stained with Bielschowsky's silver impregnation by point sampling using a 25-point Zeiss eyepiece (Brück *et al.*, 1997). All measurements were performed at 800-fold magnification. Random points were superimposed on the plaques and the periplaque white matter. The number of points crossing axons was measured as a fraction of the total number of points of the stereological grid. Axonal density within the lesion was compared with the normal appearing white matter adjacent to the plaque. The degree of axon reduction in the lesion is given as the percentage of axon density compared with the normal appearing white matter.

The number of specifically stained cells, for example CD3-positive T lymphocytes or TNF-α mRNA-positive cells, was determined per square unit of tissue on serial sections selected according to the demyelinating activity within the plaques (see above). The number of cells was counted in at least 10 standardized microscopic fields of 10 000 μm^2 each defined by an ocular morphometric grid. From these 10 areas, the mean number of cells per mm^2 is given in the text and figures.

Statistics

For statistical analysis, non-parametric tests were applied (Mann–Whitney test, Spearman rank correlation). In cases of multiple comparisons, a Bonferroni adjustment was performed. All tests were classified as significant if the P-value was <0.05. The GraphPad PRISM© software was applied for statistical calculations (GraphPad Software, Inco., San Diego, Calif., USA).

Results

Axonal injury and demyelinating activity

Axonal injury was quantified in 88 brain biopsy samples from 42 patients. Demyelinating activity was classified according to the presence of myelin proteins in macrophages (EA and LA), the presence of remyelination (ERM and LRM) and the absence of both active demyelination and remyelination (inactive demyelinated, DM) (Brück *et al.*, 1995). Demyelinating activity was heterogeneous in each biopsy specimen. Within the 88 biopsy samples investigated, a total of 109 different lesion areas were identified according to their demyelinating activity [n = 24 (EA), 20 (LA), 24 (DM), 28 (ERM) and 13 (LRM)].

Axonal density was reduced in most lesions compared

Fig. 1 Bielschowsky's silver impregnation of the normal appearing white matter (**A**) and of an actively demyelinating lesion with axon reduction (**B**). An active demyelinating lesion as indicated by the presence of PLP-containing macrophages (**C**, arrows). There are numerous APP-positive axons (**D**, arrows) present within the lesion. An inactive demyelinated lesion with macrophages containing PAS-positive degradation products (**E**, arrows). There are still a few APP-positive axons (**F**, arrows) detectable. A remyelinating lesion with uniformly thin myelin sheaths indicative of remyelination (**G**) with ongoing axonal injury as shown by the presence of APP-positive axons (**H**, arrows).

with the adjacent normal appearing white matter (Figs 1 and 2). Only single early active demyelinating and early remyelinating lesions showed axon densities within the range of the normal appearing white matter. Overall, the medians of axon reduction were similar between lesions of different demyelinating activity with a large inter-individual variability (Fig. 2A). The only significant difference was a less pronounced reduction of axonal density in early compared with late active demyelinating lesions ($P < 0.05$; Fig. 2A).

Acute axonal damage was determined in sections stained for APP. A substantial number of APP-expressing axons were identified not only in active demyelinating, but also in remyelinating lesions (Figs 1 and 2B). In these lesions, APP expression was significantly higher than in the normal appearing white matter ($P < 0.05$). Some axons in the normal appearing white matter also expressed APP. Between lesions of different demyelinating activities, there were no significant differences in APP expression. To exclude that the close vicinity of remyelinating lesions to areas of active demyelination resulted in the high numbers of APP-positive axons in areas of remyelination, cases with remyelination were studied in detail. Five cases revealed remyelinating lesions close to active demyelinating areas, whereas 15 cases only showed early or late remyelination without indication of active myelin breakdown in the vicinity. The number of APP-positive axons in these two groups (345 ± 304.9 versus 500.5 ± 846.2) did not show a statistically significant difference.

There was only a weak correlation between axonal density and APP expression within the lesions ($r = –0.22$, $P = 0.017$). APP expression and the extent of axon reduction compared with the normal appearing white matter were not significantly correlated.

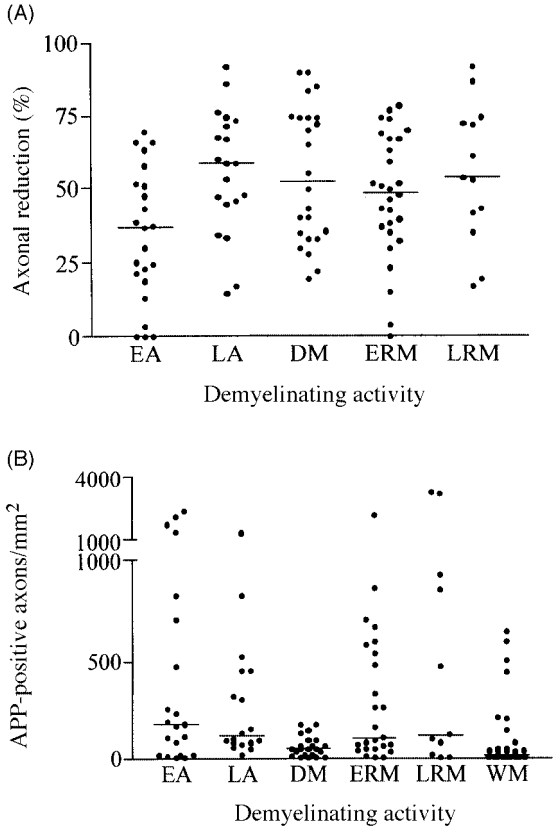

Fig. 2 (**A**) Axon reduction compared with the normal appearing white matter. Significant differences exist only between early (EA) and late (LA) active demyelinating lesions ($P < 0.05$). (**B**) APP expression in multiple sclerosis lesions of different demyelinating activity [early (EA) and late (LA) active demyelinating lesions, inactive demyelinated lesions (DM), early (ERM) and late (LRM) remyelinating lesions] and in the periplaque white matter (WM). All demyelinating and remyelinating lesions have significantly more APP positive axons than the periplaque white matter (EA, LA, ERM, LRM versus WM $P < 0.05$). Comparisons between lesion types yield no significant differences. (median)

Axonal injury and inflammation

The different cell types that constituted the inflammatory infiltrate were identified by immunocytochemistry and enumerated by light microscopy. Cell numbers were correlated with the extent of axonal loss and APP expression inside the same lesion on adjacent slices. There was no statistically significant correlation between any cell type and the relative axon density or the extent of its reduction compared with the normal appearing white matter. The number of APP-positive axons significantly correlated with the number of CD8-positive T cells ($P = 0.007$, $r = 0.29$; Fig. 3A) and cells of the macrophage/microglia lineage ($P = 0.0001$, $r = 0.44$; Fig. 3B). Additionally, the number of cells expressing major histocompatibilty complex (MHC) class II molecules, which belong predominantly to the macrophage/microglia lineage (Bö *et al.*, 1994), correlated with APP expression ($P = 0.0009$, $r = 0.32$). No significant correlation was found between CD3-positive T lymphocytes and APP-positive axons.

Axonal injury and inflammatory mediators

To define the role of TNF-α and nitric oxide, which are both candidate mediators of demyelination (Selmaj and Raine, 1988; Mitrovic *et al.*, 1994), cells expressing mRNA specific for TNF-α and iNOS were enumerated and correlated with relative axonal density, the extent of axonal loss compared with the periplaque white matter and APP expression. A separate study revealed a clear association between the number of TNF-α mRNA-expressing cells and active demyelination, whereas no such correlation was found for iNOS mRNA-positive cells (data not shown). There was a significant correlation between axon density and the number of iNOS-expressing cells but not with TNF-α-expressing cells ($P = 0.004$, $r = –0.29$). No correlation was found between either iNOS or TNF-α expression and axonal loss or the number of APP-positive axons. Steroid treatment prior to biopsy in 13 patients did not lead to a statistically significant decrease of inflammatory cell numbers or TNF-α/

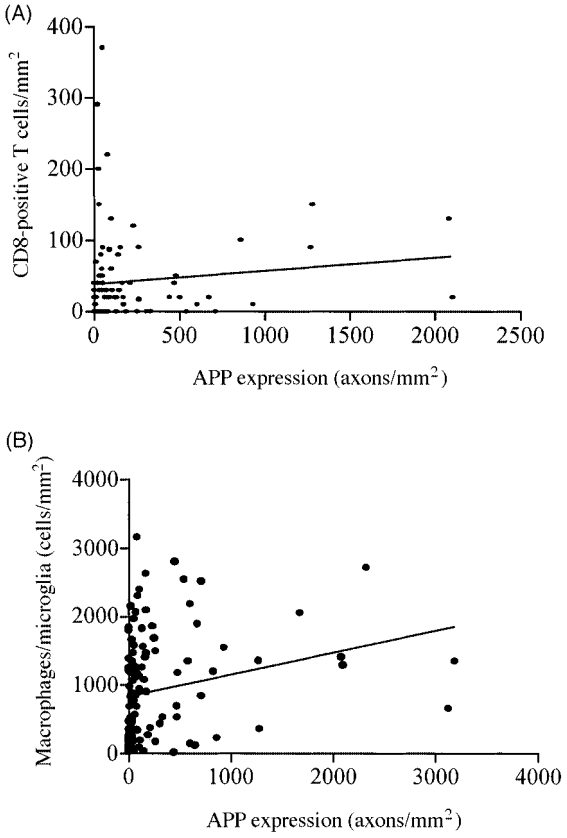

Fig. 3 (**A**) Correlation of APP expression with the number of CD8-positive cells [$P = 0.0007$; $r = 0.29$ (Spearman rank correlation)] and (**B**) cells of the microglia/macrophage lineage ($P = 0.0001$; $r = 0.44$).

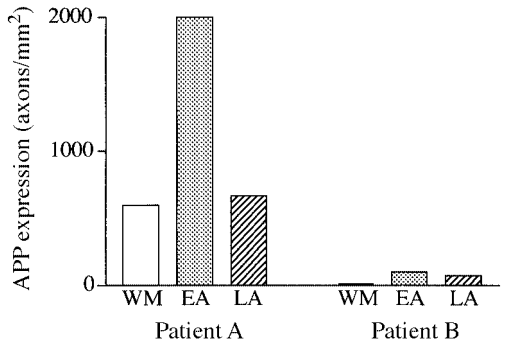

Fig. 4 APP expression in the periplaque white matter and multiple sclerosis lesions of different demyelinating activity in two distinct patients (WM = periplaque white matter, EA = early active demyelinating lesions, LA = late active demyelinating lesions). Patient A: secondary progressive course, biopsy 7 months after clinical disease onset. Patient B: monophasic course, biopsy 4 months after clinical disease onset.

iNOS mRNA expression within the lesions compared with lesions from untreated patients.

Interindividual variability of axonal injury

Both axonal loss and APP expression revealed large interindividual variability (Fig. 4A and B). Among the different lesions of one single patient, the numbers of APP-expressing axons were similar, being on either a high or a low level. This was not attributable uniformly to one or the other clinical feature, although APP expression was more pronounced in lesions from patients with a secondary progressive disease course than in relapsing–remitting or monophasic patients ($P < 0.01$; Fig. 5). The latter had only one bout with partial remission and no further exacerbation during the follow-up, which was rather short in most cases. Axonal loss and APP expression were not higher in patients with a longer pre-biopsy disease duration. In contrast, there was a trend towards a higher APP expression in the periplaque white matter of patients in the early disease stages ($P = 0.1$, $r = -0.32$). Steroid treatment prior to biopsies in 13 patients had no statistically significant effect on axon pathology. Lesions from these patients did not differ significantly from lesions of patients without steroid treatment with regard to relative axon density, axonal loss or APP expression.

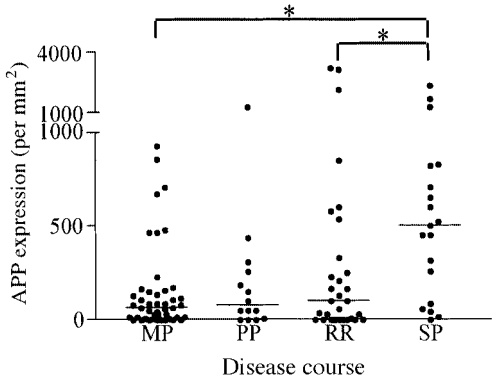

Fig. 5 Acute axonal damage and clinical disease course (MP = monophasic, PP = primary progressive, RR = relapsing–remitting, SP = secondary progressive). A monophasic course indicates one bout with complete or partial remission and no further exacerbation during the observation period. One patient with acute multiple sclerosis (Marburg) is not included. (median; $^*P < 0.05$)

Discussion

The current study on axonal pathology in biopsy tissue from multiple sclerosis lesions focuses on the coherence of axon destruction and demyelination as well as on the pathogenesis of axonal injury. The data are of course limited as they give only a snapshot of the disease process, and causal relationships between different findings cannot be proven by statistical correlation. However, beyond these limitations, the use of this large collection of human biopsy material allows the analysis of tissue during the active stages of the disease, in contrast to autopsy tissue, which often lacks active lesions with ongoing tissue destruction (Lucchinetti and Rodriguez, 1997). The tissue taken in the early course of a chronic disease may provide an answer to the question of whether axon pathology is a critical event in the formation of multiple sclerosis lesions, or just a consequence of demyelination in later plaque development. The use of strict criteria for the

classification of lesions with respect to their demyelinating activity guarantees the exact definition of the lesion stage within a narrow time window. The staining of myelin proteins in macrophages offers the most reliable approach to lesion classification because the destruction of myelin resembles myelin phagocytosis and not infiltration of inflammatory cells alone (Brück *et al.*, 1995; Lassmann *et al.*, 1998).

Studies on tissue destruction in multiple sclerosis hitherto have focused predominantly on demyelination, although the damage to axons had been described from the beginning of multiple sclerosis research (Charcot, 1868). The destruction of axons was thought to be a feature of chronic multiple sclerosis in its later stages, when the disease has entered a progressive course. Recently, a study on autopsy tissue revealed that axonal transection was even a feature of the early stages of the disease and also appeared early during lesion evolution (Trapp *et al.*, 1998). The number of transected axons was higher in lesions with a large inflammatory infiltrate and smaller in the hypocellular lesion centres (Trapp *et al.*, 1998). However, patients in this study either had acute fulminant disease, which is known to be associated with severe tissue destruction (Lucchinetti *et al.*, 1996), or died years after disease onset during the progressive course. In this study, only demyelinated axons were transected. From this, it appears likely that demyelination is a prerequisite for axonal injury, and those inflammatory mechanisms leading to demyelination may also be responsible for axonal damage.

The current study gives evidence from human material that axonal injury in multiple sclerosis is, at least in part, independent of demyelination and mediated by macrophages, microglia and CD8-positive T lymphocytes. Additionally, the present data confirm and complete earlier studies that indicated that axonal injury in chronic multiple sclerosis was an early event during disease course and lesion formation. The results do not exclude but render unlikely the direct involvement of CD4-positive T lymphocytes or of the inflammatory mediators TNF-α and nitric oxide as part of the axon-damaging process, although these cells and mediators are highly likely to induce demyelination (Selmaj and Raine, 1988; Mitrovic *et al.*, 1994).

There was a large inter-individual variability of axonal pathology, which could, in part, be attributed to the clinical course with secondary progression being associated with the most pronounced axonal damage. This is in agreement with some, but not all, magnetic resonance spectroscopy studies that showed levels of NAA, a marker of axon reduction, to be lower in secondary progressive than in relapsing–remitting multiple sclerosis (Matthews *et al.*, 1996). However, even within one group of patients with an identical clinical course, there were substantial differences in the extent of axonal injury. This indicates that other factors affect the damage of axons, factors which still have to be identified and may be related to pathogenetic heterogeneity (Lucchinetti *et al.*, 1996). Neither age at disease onset nor disease duration before biopsy had a statistically significant effect in our study, which argues against the later stages of the disease being most critical with respect to axonal loss. Surprisingly, patients with primary progressive multiple sclerosis revealed only minor signs of acute axonal damage within the lesions. Although there is no evidence of a more pronounced axonal damage in the brain from imaging studies (Davie *et al.*, 1997), spinal cord atrophy as a final consequence of brain substance loss was shown to be more extensive in primary progressive multiple sclerosis patients (Stevenson *et al.*, 1998).

To define the dynamics and the mechanisms of axonal injury in multiple sclerosis lesions, it is most important to apply a technique that allows the identification of axons that underwent transection recently, i.e. during the last several days. It is well known that inflammatory reactions and myelin degradation may occur and change within a few days (Lassmann, 1983). Therefore, it is not useful to correlate any of these features to signs of axonal damage such as the quantitative loss of axons or the appearance of axon spheroids that may have occurred months or years before. Moreover, the sole reduction of relative axon densities compared with the normal appearing white matter adjacent to the plaque is imprecise. The normal appearing white matter, especially that next to the plaque, may also be affected by axonal loss, as shown by magnetic resonance spectroscopy studies with reduced NAA levels (van Walderveen *et al.*, 1998). Moreover, interstitial oedema and cellular infiltrates may have a major influence on the extent of axonal density. These factors can, in part, explain the extreme variability of axon densities and axonal loss between patients and the lack of a correlation with numbers of inflammatory cells.

Studies of severe head trauma and ischaemic cerebral infarction demonstrated that the accumulation of APP in the proximal ends of transected axons is restricted to a period of <30 days after an acute injury and, therefore, is well suited as a marker of acute axonal damage (Li *et al.*, 1995; Pierce *et al.*, 1996; Bramlett *et al.*, 1997; Yam *et al.*, 1997). APP appears in close proximity to the site of axon transection, as shown in models of ischaemic brain infarction, and accumulates in the proximal terminal ends of axons (Ohgami *et al.*, 1992; Yam *et al.*, 1997). APP is more sensitive than conventional silver staining for the detection of axonal damage (Gentleman *et al.*, 1995). However, it is still a matter of debate if APP accumulation in injured axons resembles an irreversible state or whether at least some APP-positive axons may undergo regeneration (Ferguson *et al.*, 1997).

In the current study, APP expression as an indicator of acute axonal damage was identified during all stages of demyelination and remyelination and also in some areas of the normal appearing white matter. The high numbers of APP-positive axons in remyelinating lesions was not related to the presence of active demyelinating lesions in their close vicinity, which otherwise could easily have explained this observation. Our data, in contrast, favour axonal damage occurring independently of active demyelination, as shown above, although it cannot be ruled out that the investigated biopsy material missed focal areas of ongoing demyelination

in the vicinity of the remyelinating lesions. Therefore, axonal injury appears to be, at least in part, independent of demyelination, as axons in the normal appearing white matter and in remyelinating lesions have their myelin sheaths–although there is evidence of myelin damage in normal appearing white matter from ultrastructural studies, and myelin sheaths in remyelinated lesions are thin. The only conclusion that can be drawn from light microscopy is that axons do not have to be completely demyelinated in order to be damaged.

Despite an uncertain degree of overlap, the mechanisms involved in axonal injury may be partially different from those of demyelination. The correlation between APP expression and the number of macrophages, microglia and CD8-positive T lymphocytes suggests that inflammation is important, not only in demyelination, but also in axonal injury, which is in agreement with the findings of others (Ferguson *et al.*, 1997). However, we did not find a correlation with the whole CD3-positive T cell population but only with CD8-positive T lymphocytes. Morphologically, these CD8-positive cells resemble small round lymphocytes and do not possess the morphological characteristics of activated microglial cells, which are known to be able to express the CD8 antigen. We therefore believe that the CD8-positive cells in the demyelinating lesions are cytotoxic T cells. Further evidence for the involvement of CD8-positive T lymphocytes comes from experimental data on MHC class I-deficient mice. These animals develop demyelination but no axonal damage after infection with the Theiler's virus, and CD8-positive T cells are absent from the lesions, in contrast to normal animals (Rivera-Quiñones *et al.*, 1998).

As in demyelination, macrophages and microglia, which were not differentiated from each other in this study, also play a role in axonal injury (Brück *et al.*, 1996). The expression of MHC class II molecules as a marker of macrophage and microglial activation in multiple sclerosis lesions correlated with acute axonal injury as revealed by APP expression. One possible mechanism of macrophage involvement may be antibody-mediated destruction of axons via Fc or complement receptors. Autoantibodies against components of the axon membrane were identified in sera, cerebrospinal fluid and CNS lesions of multiple sclerosis patients (Sloviter *et al.*, 1996; Rawes *et al.*, 1997; Genain *et al.*, 1999). Antibody- and complement-mediated tissue destruction is a known feature of a subtype of multiple sclerosis (Lucchinetti *et al.*, 1996; Storch *et al.*, 1998).

Two major inflammatory mediators, TNF-α and nitric oxide, which have been identified in multiple sclerosis lesions and are largely produced by macrophages/microglia, are suspected to mediate demyelination (Selmaj and Raine, 1988; Mitrovic *et al.*, 1994). The lack of a correlation with signs of acute axonal injury gives no evidence for their involvement in axon pathology. The low axon density in lesions with increased iNOS expression may be a consequence of oedema caused by nitric oxide-mediated blood–brain barrier damage (Giovannoni, 1998).

The findings from this study have implications for the current understanding of multiple sclerosis pathology, pathogenesis and treatment strategies. Demyelination and axonal injury are at least partially distinct events with different mechanisms involved. Both occur early in the disease course, even in chronic multiple sclerosis. Demyelination is reversible and does not necessarily lead to a complete conduction block. In contrast, axon transection peremptorily results in a conduction block and there has not been any evidence so far of axon regeneration after transection in the CNS. Therapies must therefore aim at preventing not only demyelination, but also axonal injury. The fact that most patients that are treated with interferon-β or copolymer-1 still progress may be suggestive not only of ongoing demyelination but also of axonal injury, which is perhaps not being treated sufficiently by these agents. Also, therapeutic strategies that are candidates for inducing remyelination, such as intravenous immunoglobulin, cannot be expected to prevent axonal injury, as this occurs also in remyelinated lesions. In order to evaluate the effect of therapeutic agents on axonal injury, *in vivo* markers of axonal loss, such as the quantification of NAA or cerebral and spinal atrophy, need to be implemented into clinical studies.

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