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Axonal damage in acute multiple sclerosis lesions

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Summary

One of the histological hallmarks of early multiple sclerosis lesions is primary demyelination, with myelin destruction and relative sparing of axons. On the other hand, it is widely accepted that axonal loss occurs in, and is responsible for, the permanent disability characterizing the later chronic progressive stage of the disease. In this study, we have used an antibody against amyloid precursor protein, known to be a sensitive marker of axonal damage in a number of other contexts, in immunocytochemical experiments on paraffin embedded multiple sclerosis lesions of varying ages in order to see at which stage of the disease axonal damage, in addition to demyelination, occurs and may thus contribute to the development of disability in patients. The results show the expression of amyloid precursor protein in damaged axons within acute multiple sclerosis lesions, and in the active borders of less acute lesions. This observation may have implications for the design and timing of therapeutic intervention, one of the most important aims of which must be the reduction of permanent disability.

Keywords: multiple sclerosis; amyloid precursor protein; APP; axon; damage; lesion

Abbreviation: APP = amyloid precursor protein

Introduction

Historically the detection of axonal injury in acute multiple sclerosis has been difficult. Multiple sclerosis lesions are typified by leucocyte infiltration and demyelination in characteristic locations. The degree of axonal loss is commonly assessed by silver stains. A new technique which depends on immunoreactivity for amyloid precursor protein (APP) in axons has been shown to be a sensitive method, in formalin-fixed paraffin embedded sections, for detecting diffuse axonal injury following head injury (Gentleman *et al*., 1993, Sherriff *et al*., 1994). We have used this method to detect whether there is axonal damage associated with the early lesions of multiple sclerosis.

APP is normally present in neurons as well as giving rise to beta-amyloid deposits in the brain in Alzheimer's disease. APP is known to be transported by fast axonal transport (Koo *et al*., 1990) and there is evidence that it is associated with the endosomal/lysosomal system (Haass *et al*., 1992; Ferreira *et al*., 1993). In injured axons, the cytoskeleton breaks down, possibly due to calcium influx (Adams *et al*., 1991), causing interruption of axoplasmic flow and subsequent accumulation of organelles (Povlishock, 1992). Normal levels of axonal APP are not detectable by standard immunocytochemistry in formalin-fixed tissue, and its detection by this method at sites of axonal injury is thought to represent accumulation of APP due to failure of axonal transport.

Material and methods

Eighteen cases of multiple sclerosis and five cases of normal brain were used in the study. The paraffin blocks for the study were obtained from the collections of post-mortem brain tissue in the Neuropathology Department at the Radcliffe Infirmary, Oxford and from the Corsellis Collection at Runwell Hospital, Essex. All the tissue blocks had been fixed in 10% formalin and embedded in paraffin wax.

Immunocytochemistry

Sections, 10 μm thick, were cut on a microtome and picked up on gelatinized slides. Sections were dewaxed and endogenous peroxidase activity was blocked by incubation with 1% H_2O_2 ethanol for 30 min. The sections were then microwaved in 0.1 M citric acid buffer (pH 6) bringing them to the boil, then cooling them for 5 min and then reboiling them and allowing them to cool for 20 min, prior to immunohistochemical staining.

The binding of the primary antibodies was revealed by an avidin–biotin–peroxidase method as previously described (Lawson *et al*., 1990), with reagents supplied by Vector Laboratories (Peterborough, UK). Briefly the method was as follows. Microwaved sections were rinsed in 0.1 M PBS/Tween (phosphate buffered saline containing 0.1% Tween 20) and incubated in 10% foetal calf serum for 30 min. After 30 min the excess serum was removed and the sections were incubated with primary antibody for 2 h. The primary antibodies used and their dilutions are listed below. The sections were washed in PBS/Tween and incubated with biotinylated horse anti-mouse IgG (1:100) for 45 min. After rinsing in PBS/Tween, the sections were incubated in avidin–biotin complex for 45 min. The horseradish peroxidase was visualized with 3′3′-diaminobenzidine solution containing 0.25% H_2O_2. Negative controls omitting the primary antibody were used for each case. All sections were counterstained with haematoxylin and eosin, dehydrated and coverslipped.

Primary antibodies and their dilutions used in this studies were: CD3 (Anti-T cells, 1:100; Dako), PGM1 (Anti-CD68, 1:200; Dako), LN27 (Anti-APP, 1:50; Zymed), 22C11 (Anti-APP, 1:100; Boehringer).

Classification of multiple sclerosis lesions

In addition to immunohistochemical staining, multiple sclerosis lesions were stained with Luxol Fast Blue to reveal the extent of myelin loss, and Palmgren's silver stain for axons. The multiple sclerosis lesions were divided into three categories: acute, active chronic and chronic. Acute lesions were those with numerous T cells (CD3+ cells) and macrophages (CD68+ cells) throughout. They were further subdivided into those lesions with Luxol Fast Blue inclusions in macrophages and those without. In active chronic lesions, the centre contained few macrophages and T cells but there were more of these cells at the border between demyelinated and normally myelinated tracts. Chronic lesions were those with few or no macrophages and T cells in the centre or periphery of the lesion.

Quantitative analysis of APP immunoreactivity macrophage numbers in multiple sclerosis lesions

Both APP positive (APP+) elements and macrophages were counted under a ×100 objective. In each section, macrophages and APP+ elements were counted across the entire lesion in a 0.1 mm wide strip taken through the middle of the lesion. The numbers of APP+ elements and macrophages counted in areas of 0.01 mm^2 were plotted. Data were plotted separately for each lesion. In addition, mean values of APP and macrophage counts were calculated for each lesion. In active chronic lesions the border and the centre of the lesion were analysed separately. APP data were evaluated with a two-tailed Student's *t* test using Abacus Concepts, StatView (Abacus Concepts, Berkeley, Calif., USA).

Results

Nineteen lesions from 18 cases were studied. The results showed that APP expression is most pronounced in the most acute lesions. The APP staining was of two different types. It stained elements in a round spheroid-like shape with an approximate diameter of 40–80 μm or in a beaded string-like appearance along the length of an axon. In longitudinal or oblique section, some of the APP-reactive profiles were carrot-shaped or irregular where axons were swollen.

Acute lesions

Six acute lesions from five cases of multiple sclerosis were studied. Figure 1A and B shows a typical example of an acute multiple sclerosis lesion stained for APP and macrophages, respectively. There was a good correlation between the number of macrophages and the extent of axonal damage (Fig. 2A). The acute lesions contained the most numerous APP+ axons and macrophages (Fig. 3). The average density of APP+ elements in acute lesions was similar to that at the border of active chronic lesions, but it was significantly higher ($P < 0.001$) than in the centre of active chronic and in chronic multiple sclerosis lesions.

Acute lesions that contained Luxol Fast Blue inclusions in macrophages, indicating ongoing demyelination, contained more APP+ axons than acute lesions that lacked these inclusions. It was not possible to determine the percentage of axons that were stained but within each lesion there were hundreds of positively stained axons. The staining was seen throughout the lesion, but hardly at all in surrounding white matter. Additional silver staining on the adjacent sections showed the presence of many axons in acute lesions some of which had abnormal profiles.

Active chronic lesions

Six active chronic multiple sclerosis lesions from six different cases of multiple sclerosis were studied. APP axonal staining was found closely related to the areas of these lesions that contained active macrophages stained with PGM1 antibody and was not found elsewhere (Fig. 1C and D). As shown in Fig. 2B, APP staining was found predominantly at the border of these lesions and correlated well with the number of macrophages in these areas. However, there was significantly less staining ($P = 0.002$) in the centre of these lesions (Fig. 3A). Although there was a variable number of APP+ axons in the active border zones, in general they were present in as great a density as in acute lesions, albeit in a more restricted location. Silver staining showed that some axons were still present in the centre of these active chronic lesions but the density was reduced when compared with the normally myelinated adjacent tissue.

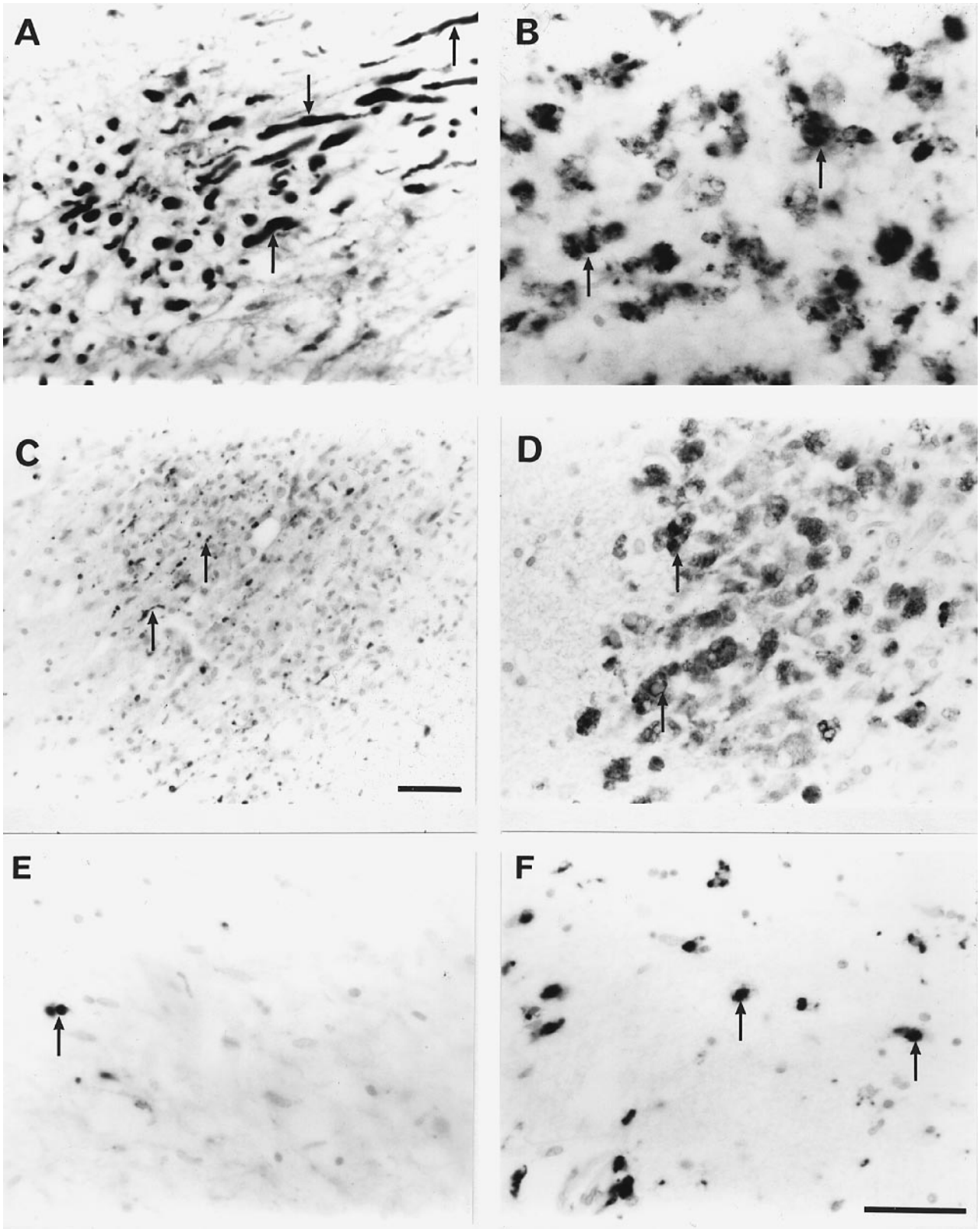

Fig. 1 APP immunoreactivity in multiple sclerosis lesions. (**A** and **B**) Acute multiple sclerosis lesion; staining with LN27 monoclonal antibody (anti-APP) (**A**) shows many APP+ axons (arrows) inside the lesion and staining with PGM1 monoclonal antibody on the adjacent section (**B**) shows the extent of macrophage (arrows) infiltration in this lesion. (**C** and **D**) Active chronic lesion; a low power micrograph shows many APP+ axons (arrows) at the border of a multiple sclerosis lesion (**C**) and foamy macrophages associated with the active multiple sclerosis border are shown in **D**. (**E** and **F**) Chronic multiple sclerosis lesion; the extent of APP staining (**E**) and the number of macrophages (**F**) in this lesion is very small. Scale bars represent 50 μm. **A**, **B**, **D**, **E** and **F** are of the same magnification.

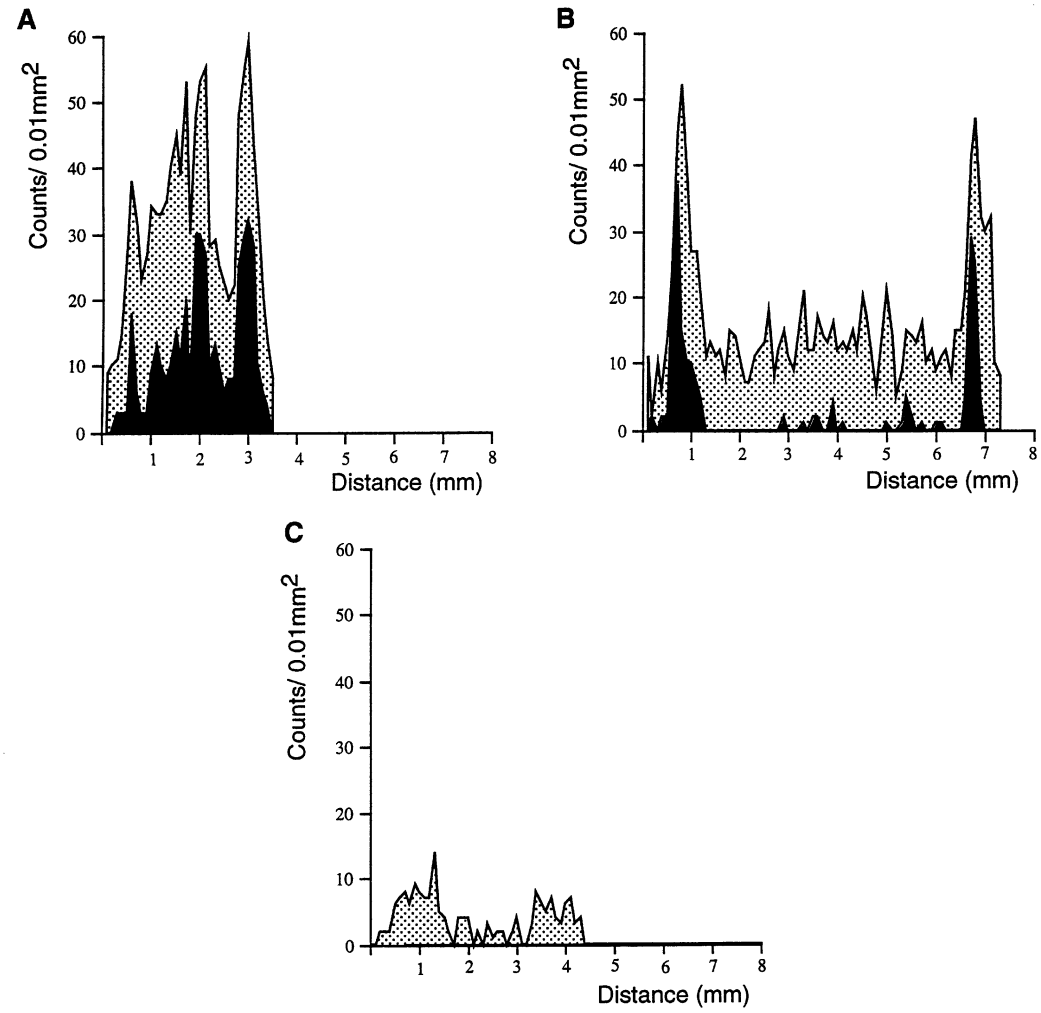

Fig. 2 Graphs to illustrate the profile of axonal injury (APP, stippled areas) and the number of macrophages (black areas) in a typical acute multiple sclerosis lesion (**A**), active chronic multiple sclerosis lesion (**B**) and chronic multiple sclerosis lesion (**C**).

Chronic lesions

Seven lesions from six different cases of multiple sclerosis were studied. These lesions were typified by low levels of staining for PGM1 (Fig. 1F) and CD3. There was also almost no staining with anti-APP antibody (Fig. 1E). Figure 2C shows a typical profile of a chronic lesion, there were a few areas at the margins of some chronic lesions which contained small numbers of PGM1+ foamy macrophages and within these areas there was a low level of axonal APP staining. Silver stains showed that these chronic lesions had a markedly reduced content of stainable nerve fibres.

Discussion

The results from this study show that APP, when used as a marker of early axonal damage in multiple sclerosis lesions, is expressed in areas of acute inflammation and demyelination and not in the more chronic areas of the lesions. The histology of multiple sclerosis has been studied for over 100 years but the literature on axonal damage in multiple sclerosis is confusing. To some extent this reflects technical limitation of silver stains on embedded material, which are inadequate for demonstrating all surviving axons, and the difficulty in positively identifying degenerating axons in plaques. However, there are two relevant observations that have been made on many occasions in the older literature on multiple sclerosis (Charcot, 1877; Dawson, 1916; Buzzard and Greenfield, 1921; Greenfield and King, 1936; Putnam, 1936; Adams and Kubik, 1952; Peters, 1968; Shintaku, *et al.*, 1988). The first is that some abnormal axons can be identified in both acute and chronic lesions and the second is that Wallerian degeneration in multiple sclerosis is largely confined to long-standing cases. A further implication that follows is that some of the damage seen in acute lesions is possibly reversible (Peters, 1968). The time course over which axon damage develops in long-standing multiple sclerosis is not at all clear, e.g. whether it accrues steadily over years or whether it occurs episodically and in relation

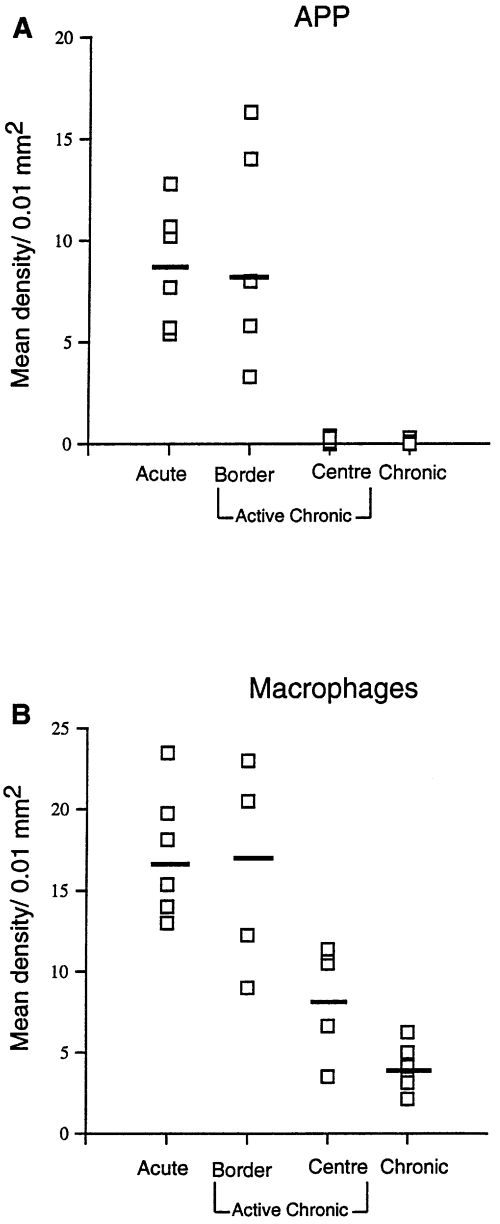

Fig. 3 Scatter plots showing mean density of APP+ axons (**A**) and macrophages (**B**) in all lesions studied. Each square represents one lesion. Note that there is almost no APP reactivity in the centre of active chronic lesions and in chronic lesions. The average number of injured axons (bar) in acute lesions is similar to that at the border of active chronic lesions. Likewise the average number of macrophages is similar in these two areas.

to episodes of demyelination. There seems at the present time to be some importance in distinguishing between the possibilities and in attempting to understand the pathogenesis of axonal damage, since it is widely agreed that it is likely to determine the severity of the permanent neurological deficits that develop in multiple sclerosis (Barnes *et al*., 1991; McDonald *et al*., 1992; Filippi *et al*., 1994). Thus, interventions that affect the number of relapses of multiple sclerosis suffered may have more influence on the survival of axons if damage to them results from processes associated with relapse than if damage is independent of these processes.

The present study used a novel and sensitive method of detecting axon damage that relies on the accumulation of immunoreactive APP, a protein normally transported by fast axonal transport, in damaged axons. In a recent study of axonal damage following human head injury, the method was shown to be much more sensitive than silver staining for detecting damaged axons and it was concluded that silver staining greatly underestimated the frequency of axonal damage after head injury (Gentleman *et al*., 1995). In our study of multiple sclerosis the method revealed an unexpected wealth of damaged axons throughout acute plaques and at the margins of active chronic plaques.The close localization in relation to the areas containing inflammation, active demyelination and macrophages was striking and suggests that axonal damage, like demyelination in multiple sclerosis is closely associated with inflammation. The difficulty in interpreting the significance of the axonal APP immunoreactivity is that it is not certain whether it is compatible with reversibility of damage. The swollen ends of the axons are, however, characteristic of end bulbs following axon transection. Other studies of axonal damage in human material detected with this method have been made chiefly in conditions well recognized to be associated with irreversible axonal damage such as severe head trauma (Sherriff *et al*., 1994; Gentleman *et al*., 1995) and cerebral infarction (Cochran *et al*., 1991; Ohgami *et al*., 1992), but even in these situations it might be possible for some axons to be reversibly damaged alongside others that are irreversibly damaged.

Some experimental studies in animals of axonal APP immunoreactivity have cast some light on the time course of development of this feature following brain or spinal cord injury. Following contusional injury to rat brain such immunoreactivity was intense at 1–3 days and was detectable, but less intense, at 21 days after lesioning (Lewen *et al*., 1995). After rat spinal cord compression it was present for 4 h to 9 days following which the experiment was terminated (Li *et al*., 1995). In human disease, apart from head injury in which it is an early marker of injury, it is harder to relate expression of axonal APP immunoreactivity to the age of lesions, but in adult white matter vascular lesions it was found in mild or early lesions but was absent from the centre of older or more severe lesions (Suenaga *et al*., 1994). Likewise, in periventricular leukomalacia in infants it was thought to label the early stages of lesion development (Arai *et al*., 1995). The time period over which a damaged axon appears APP+ will, of course, influence the apparent amount of axonal damage. If APP+ staining is a short-lived event, it will result in an underestimate of the amount of axonal damage. This has obvious parallels with estimates of cell death from the relatively short time in which a cell appears apoptotic.

While acknowledging the uncertainty about the reversibility of axonal damage detected by APP immunoreactivity, it seems at least possible that some of the axons so labelled in

acute and active chronic multiple sclerosis may be irreversibly damaged and provide the underlying substrate for long-standing axonal depletion. If the alternative possibility, that axonal damage develops gradually and progressively during the course of the disease,were correct (McDonald *et al*., 1992), our study might have been expected to show APP immunoreactive axons in chronic plaques. In fact we did not find this and the little APP immunoreactivity associated with chronic plaques was entirely confined to marginal areas with a mild, focal, macrophage reaction. Thus, we interpret our findings as favouring episodic axonal damage closely linked to inflammation and relapse in multiple sclerosis, albeit with some reservations. In the light of our findings it may be worth re-examining the evidence that has led to multiple sclerosis being thought of as an essentially demyelinating disease until relatively late in its course; in particular, further more definitive studies of axon reactions should be undertaken.

The only previous study of APP immunoreactivity in multiple sclerosis of which we are aware is that by Gehrmann *et al.* (Gehrmann *et al*., 1995). This study differed somewhat from the present one, both methodologically and in its focus and findings; it was based on examination of frozen sections and focused principally on expression of APP in inflammatory cells and neuroglia cells. In frozen sections APP was detected in normal brain to a low extent in blood vessel walls, oligodendrocytes, resident microglial cells and neurons. In actively demyelinating plaques, its expression was increased and it was detected in T lymphocytes, foamy macrophages, activated microglia and reactive astrocytes. In chronic lesions, mention is made of a dense network of APP-positive astrocytic processes and demyelinated axons, but the axonal pattern is not described in any detail. Clearly the pattern of APP expression was very different from that described in formalin-fixed paraffin embedded sections in this and other studies.

The main focus for treatment of multiple sclerosis must be the reduction of permanent disability. It is accepted that permanent disability results from cumulative axon loss. If this axon loss is occurring very early in the disease and effective treatment is available, the timing of therapeutic intervention should reflect the timing of the start of axonal damage. This would mean treatment starting early, not being based on the severity of the clinical state of the disease but on preventing the cumulative assaults which result eventually in permanent disability.

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