T-cell- and macrophage-mediated axon damage in the absence of a CNS-specific immune response: involvement of metalloproteinases

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Summary

Recent evidence has highlighted the fact that axon injury is an important component of multiple sclerosis pathology. The issue of whether a CNS antigen-specific immune response is required to produce axon injury remains unresolved. We investigated the extent and time course of axon injury in a rodent model of a delayed-type hypersensitivity (DTH) reaction directed against the mycobacterium bacille Calmette-Guérin (BCG). Using MRI, we determined whether the ongoing axon injury is restricted to the period during which the blood–brain barrier is compromised. DTH lesions were initiated in adult rats by intracerebral injection of heat-killed BCG followed by a peripheral challenge with BCG. Our findings demonstrate that a DTH reaction to a non-CNS antigen within a CNS white matter tract leads to axon injury. Ongoing axon injury persisted throughout the 3-month period studied and was not restricted to the period of blood–brain barrier breakdown, as detected by MRI enhancing lesions. We have previously demonstrated that matrix metalloproteinases (MMPs) are upregulated in multiple sclerosis plaques and DTH lesions. In this study we demonstrated that microinjection of activated MMPs into the cortical white matter results in axon injury. Our results show that axon injury, possibly mediated by MMPs, is immunologically non-specific and may continue behind an intact blood–brain barrier.

Keywords: axon damage; APP; DTH; MMP; multiple sclerosis

Abbreviations: APP = amyloid precursor protein; BBB = blood–brain barrier; BCG = bacille Calmette-Guérin; DTH = delayed-type hypersensitivity; MMP = matrix metalloproteinase

Introduction

Multiple sclerosis is the archetypal inflammatory demyelinating disease of the central nervous system (Raine and Cross, 1989). The pathology of the disease is characterized by the loss of myelin from regions of CNS white matter with relative sparing of axons. Although axon loss has long been recognized (for review, see Kornek and Lassmann, 1999), the potential significance of this component of the pathology in the progression of the underlying clinical disease has been highlighted only in recent years. Evidence from both neuropathological and magnetic resonance investigations has shown that axon loss may occur early in the disease process.

The detection of axon injury has in the past depended largely on the application of silver staining methods to demonstrate loss of axons from multiple sclerosis lesions and the presence of neurofilament accumulations in axon end-bulbs formed after an axon is injured (Kornek and Lassmann, 1999). An immunocytochemical approach that relies upon the detection of amyloid precursor protein (APP) accumulation in axon end-bulbs has been shown to provide a superior and far more sensitive technique (Sherriff et al., 1994). The application of APP immunocytochemistry to multiple sclerosis lesions has shown that axon injury occurs early in the evolution of the lesions and that the extent of axon injury is closely correlated with the number of inflammatory cells (Ferguson et al., 1997; Trapp et al., 1994). Confocal microscopy was used to confirm that neurofilament accumulations in axons were indeed end-bulbs rather than swellings that simply represented an arrest of axon transport. Analysis of axon numbers in the spinal cord and corpus callosum from multiple sclerosis patients has shown that there is a profound loss of axons from these structures (Ganter et al., 1999; Evangelou et al., 2000).
The application of magnetic resonance spectroscopy also provides evidence for axonal injury in the evolving multiple sclerosis lesion. The signal from N-acetyl aspartate, widely considered to be a marker of neurones, can be used to investigate the loss of axons from major fibre tracts. It has been reported that the N-acetyl aspartate signal is reduced in both multiple sclerosis lesions and in normal-appearing white matter, the latter presumably a consequence of Wallerian degeneration of damaged axons (Davie et al., 1994; Fu et al., 1998). However, some authors have reported that the N-acetyl aspartate signal is restored as the inflammatory lesions resolve (Davie et al., 1994; De Stefano et al., 1999). The interpretation of this is complex, since it may represent the resolution of oedema, the recovery of some reversible injury to the axons or even some remyelination. A recent study reports that, when oligodendrocytes are allowed to mature in vitro in the presence of the appropriate growth factors, they may express a level of N-acetyl aspartate comparable with that found in neurones (Bhakoo and Pearce, 2000).

It is important to elucidate the cellular and molecular events that underlie the axon damage. The inflammatory infiltrate seen in multiple sclerosis lesions consists predominantly of T cells and macrophages and is consistent with the pattern of inflammation seen in the delayed-type hypersensitivity (DTH) response (Matyszak and Perry, 1995; Lassmann, 1999). Although the antigen or antigens against which this immune response is directed is a matter of debate, it is widely believed that the response is directed against antigens of the myelin sheath (Genain et al., 1999). It is not known whether the axonal injury seen in the multiple sclerosis lesion depends upon an immune response directed against a particular CNS antigen, e.g. an antigen located in the axon membrane, or whether it involves an immunologically non-specific injury that arises as a consequence of the secretion by macrophages of molecules that have been activated by T-cell cytokines, such as γ-interferon.

To address the issue of whether the immune response needs to be directed against a CNS antigen to produce axon injury, we investigated the extent and time course of axon injury in a rodent model of a DTH reaction directed against a non-CNS antigen, the mycobacterium bacille Calmette–Guérin (BCG). Injection of heat-killed BCG into the brain parenchyma results in an acute inflammatory response which rapidly resolves, after which the BCG organisms are sequestered behind the blood–brain barrier (BBB) (Matyszak et al., 1997). The immune system remains immunologically naive to this antigen in the brain (Matyszak and Perry, 1998) until the immune system is activated by a subcutaneous challenge with BCG many weeks later. The ensuing DTH response is characterized by a T-cell and macrophage infiltrate, breakdown of the BBB and tissue damage, including primary demyelination (Matyszak and Perry, 1995, 1996a).

In this study we used APP immunocytochemistry to investigate the temporal and spatial components of axon injury in this DTH response against a non-CNS antigen. We used MRI to investigate whether axon injury is restricted to the period during which the BBB is compromised by the invading leucocytes. Since we have shown previously that matrix-degrading metalloproteinases (MMPs) are upregulated in both multiple sclerosis lesions (Anthony et al., 1997) and in the DTH lesions (Anthony et al., 1998), we investigated whether these potent proteinases lead to axon injury in fibre tracts.

Material and methods

Animals
Adult (200 g) male Lewis rats (Charles River UK) were used for these experiments. All experimental procedures involving animals were carried out in accordance with the Animals Scientific Procedures Act (1986) under Home Office approval.

Reagents
All of the chemicals used in the study were supplied by Sigma (Poole, UK) unless otherwise stated. BCG was a kind gift from Dr G. Milon (Pasteur Institute, France) and was killed by boiling for 10 min before use.

Antibodies
The primary antibodies used in this study were 22C11 anti-APP (Boehringer Mannheim, Bracknell, UK). SMI31 and anti NF-H (neurofilament H) (Affiniti, Nottingham, UK). The anti-MBP (myelin basic protein) antiserum has been described previously (Matyszak and Perry, 1995).

Induction of delayed-type hypersensitivity response in the CNS

DTH responses were elicited in the CNS as reported previously (Matyszak and Perry, 1995). Briefly, Lewis rats were anaesthetized with Avertin (Sigma-Aldrich, Poole, UK). Heat-killed BCG (5 × 10⁵ organisms in a volume of 1 μl) were injected into the subcortical white matter overlying the dorsal striatum using stereotaxic surgery. Four weeks after the intracranial injection, the animals received a bilateral subcutaneous injection of 1 × 10⁷ BCG organisms in complete Freund’s adjuvant into the hind flanks. The animals were deeply anaesthetized and perfused transcardially 1 and 2 weeks and 1, 2 and 3 months after the subcutaneous challenge (n = 5 at each time point), using heparinized Tyrode’s buffer followed by Bouin’s fixative. The brains were then removed and processed for embedding in paraffin wax. An additional group of animals was injected intracranially with BCG but did not receive the subcutaneous challenge. This group was perfused 1 month after the intracranial injection.
**Stab lesions**

Lewis rats were anaesthetized as described above. Each animal was then placed in a stereotaxic frame. A midline incision was made in the scalp. A small groove was made in the skull with a dental drill, and the remaining thin layer of bone overlying the parietal cortex was removed. A fine scalpel blade was inserted into a needle-holder on the frame and then lowered into the brain using the appropriate coordinates (bregma +0.5, lateral 1.5 mm, depth 3.0 mm) to produce a lesion in the cortical white matter. The blade was withdrawn, the scalp sutured and the animal allowed to recover. Animals were killed and perfusion-fixed as described above 4, 48 and 72 h and 1 week after the lesion (n = 4 at each time point). Tissue was processed as described above.

**Microinjection of activated MMPs**

Using a minimally invasive stereotaxic procedure, 10 mM 2-aminophenylmercuric acetate-activated MMP-2, MMP-7, MMP-9 or vehicle (British Biotech, Oxford, UK) was microinjected in a volume of 1 µl (1 mg/ml) into the subcortical white matter overlying the striatum. Lewis rats were anaesthetized as described above (n = 3 per group). The microinjections were performed with a fine glass capillary with a tip diameter of <50 µm. For comparison with tissue damage in the CNS, rats (n = 3 per group) received injections of activated MMPs into the sciatic nerve. Each rat was anaesthetized and the sciatic nerve exposed at mid-thigh level. Using a fine glass capillary, activated MMP-9 (1 mg/ml) was injected in a volume of 1 µl. Twenty-four hours after the injection, the animals were terminally anaesthetized and perfused with Bouin’s fixative as described above. The brains and sciatic nerves were embedded in paraffin wax and processed as described above.

**MRI of the lesions**

In four animals, DTH responses were elicited in the CNS as described above (Matyszak and Perry, 1995). Magnetic resonance measurements were made using a 300 MHz vertical magnet as described previously (Blamire et al., 2000) and data were acquired using a Varian Inova console (Varian, Palo Alto, Calif., USA). The animals were imaged serially after the subcutaneous challenge at days 0, 3, 7, 11 and 31. To assess BBB permeability, T₁-weighted images [spin-echo sequence; TR (repetition time) = 500 ms, TE (echo time) = 20 ms] were acquired 10 min after the administration of a 100 µl bolus of a standard gadolinium-based MRI contrast agent (Omniscan; Nycomed Amersham, UK), which was injected via a tail-vein cannula. To look for image enhancement as a consequence of BBB breakdown, T₁-weighted images were also acquired prior to injection of contrast agent for comparison of pre- and post-contrast data. Images were acquired in the coronal plane (1 mm slice thickness).

**Immunocytochemistry**

Wax sections were cut and immunocytochemistry was carried out as described previously (Ferguson et al., 1997). Briefly, dewaxed sections were microwaved in citrate buffer, rinsed in 0.1 M PBS/Tween (phosphate-buffered saline containing 0.1% Tween 20) and then incubated in 10% normal horse serum for 30 min. The horse serum was removed and the sections were incubated with the primary antibodies for 2 h at room temperature. The sections were then washed in PBS/Tween and incubated with biotinylated secondary IgG (immunoglobulin G) (1 : 100) for 45 min followed by avidin–biotin–peroxidase complex for 45 min. The binding of the complex was revealed using 3,3’-diaminobenzidine solution containing 0.25% hydrogen peroxide. Negative controls omitting the primary antibody were used in each case. All sections were counterstained with haematoxylin and eosin, dehydrated and coverslipped.

Images were captured on a PC using LeicaQwin software (Cambridge, UK). Sections from representative lesions at different times in the evolution of the lesions were selected. Reconstructions of these lesions were then made using a Leitz drawing tube.

**Results**

Axon injury can be identified by the immunocytochemical localization of APP in axon end-bulbs (Sherriff et al., 1994). In order to establish how rapidly the APP-positive (APP⁺) end-bulbs appear and how long they persist using our particular immunohistochemical protocol, we first examined the white matter after a stab lesion to the cerebral white matter, in the same location as we had targeted our DTH lesions.

**APP in stab lesions**

The distribution of APP⁺ elements following acute axonal transection was examined in stab lesions at times from 4 h up to 1 week after the lesion (Fig. 1). The lesions appeared as discrete areas of damage, comprising a narrow region of necrotic tissue extending from the cortex, through the subcortical white matter and into the striatum. The tissue surrounding the lesions appeared oedematous, while the rest of the tissue appeared normal. Four hours (Fig. 1A) after the stab lesion, numerous small APP⁺ elements were present in the cortical grey matter up to 150 µm from the lesion border, while APP⁺ elements in the subcortical white matter extended up to 250 µm from the lesion edge. By 48 h (Fig. 1B) after the lesion, the number of APP⁺ elements in the cortical grey matter was greatly reduced and the most remote of these were found 100 µm from the lesion periphery. Although there was a significant reduction in the number of APP⁺ elements in the subcortical white matter, some, as large as 12 µm in diameter, were present 500 µm from the lesion site.
Seventy-two hours after the lesion, there were even fewer APP⁺ elements than at 48 h. A small number of APP⁺ elements were still present in the subcortical white matter and also in fibre tracts in the underlying striatum. They had generally increased in size; some were as large as 15 µm in diameter, and could be found up to 750 µm from the lesion periphery. By 1 week after the stab, the number of APP⁺ elements was considerably less than was seen at earlier times and the diameter of the largest APP⁺ elements had increased to 20 µm (Fig. 1C). A reconstruction of the change in APP⁺ element distribution in representative stab lesions over the time course investigated is shown in Fig. 2.

Thus, using the current fixation protocol with APP immunocytochemistry, we can detect evidence of axon end-bulb formation within 4 h after the injury. The number of end-bulbs rapidly declines over the first 48 h, indicating that, although some of the APP⁺ end-bulbs (Fig. 2) may persist...
for as long as 1 week, the majority are cleared, or resorbed by 72 h.

**DTH lesions**

The DTH lesions appeared within 1 week after the subcutaneous sensitization as a focal inflammatory lesion centred on the BCG deposit made by the intracranial injection. The cellular composition of the infiltrate has been described previously (Matyszak and Perry, 1995, 1996a). The centre, or core, of the lesion is composed predominantly of rounded mononuclear phagocytes and T cells surrounding a central vessel or vessels. Microglia, with a morphology characteristic of activated microglia, are present in the adjacent white and grey matter. Some vessels outside the major focus of the lesion are surrounded by perivascular cuffs. As a result of slight variation between injections, there are small inter-animal differences in the site occupied by the lesions.

**Neurofilament expression**

Expression of neurofilament 200 kDa has been used to visualize fibre tracts and evidence of axon damage. Transected axons in multiple sclerosis lesions have been identified by the presence of neurofilament-positive retraction bulbs (Trapp et al., 1998). The distribution of neurofilament immunoreactivity was examined in DTH lesions from 2 weeks to 3 months after their initiation by the subcutaneous challenge. An example of neurofilament immunoreactivity in a typical DTH lesion can be seen in Fig. 1. In 2-week lesions (Fig. 1D) an area packed with macrophages and T cells and devoid of neurofilament staining is surrounded by axons. A small number of retraction bulbs were observed at the lesion periphery. Lesions at 1 month occupied a larger area and the area of neurofilament loss was greater than at earlier time points (Fig. 1E). The cellular infiltrate was surrounded by a border several hundred micrometres wide, where the intensity of neurofilament immunoreactivity was significantly reduced. Axon end-bulbs up to 10 µm in diameter (Fig. 1F) were seen at the interface of the white matter and the mononuclear cell accumulation.

In lesions 2–3 months old, the area occupied by the cellular infiltrate had continued to enlarge and there was again a zone around the lesion which showed a reduction in neurofilament intensity similar to that seen in the 1-month lesions. A small number of axon end-bulbs were seen around the lesion periphery. Despite the increasing size of the lesion and the presence of axon end-bulbs, there was no clear evidence of axon loss at a distance several millimetres from the borders of the cellular infiltrate, suggesting that the increasing area of neurofilament loss was produced in part by displacement of the axons by the invading or proliferating macrophages and T cells. To confirm that the increase in lesion size was associated with ongoing axon damage rather than simply axon displacement, we examined the same lesions for ongoing or acute axon injury using APP immunocytochemistry.
APP expression
APP⁺ elements were present around the periphery of DTH lesions at all time points investigated and were absent from the rest of the brain tissue examined (Fig. 6C). The distribution of the APP⁺ elements around the lesions was found to differ as the lesions evolved. APP⁺ elements in a typical 2-week and 3-month DTH lesion can be seen in Fig. 3A and B. Lesions were examined 1 week after the subcutaneous challenge, and at this time a few APP⁺ elements were observed bordering the lesion. By 2 weeks after the subcutaneous challenge, the lesions had increased in size as more infiltrating cells accumulated. APP⁺ elements were seen adjacent to these cuffed vessels and up to 100 µm from the border of the inflammatory infiltrates. Some APP⁺ elements were also found up to 500 µm from the lesion border in the subcortical white matter, as well as around some vessels showing perivascular cuffing in the grey matter. Reconstructions of the changing distribution of APP⁺ elements in a series of representative DTH lesions at different time points are illustrated in Fig. 4A and B.

Typically, 1 month after initiation, the lesions traversed the subcortical white matter and occupied part of the overlying cortical grey matter. APP⁺ elements were found around the periphery of the lesion in the grey matter. In the subcortical white matter a high density of APP⁺ elements was present around the lesion periphery, with some larger elements (up to 15 µm in diameter) in the fibre tracts. APP⁺ elements were present 500 µm from the edge of the lesion but their density was maximal within 200 µm of the lesion border. The area occupied by the lesion 2 months after initiation was larger than at earlier times investigated. The number of APP⁺ elements was again greater than in earlier lesions and in the subcortical white matter some very large elements (up to 25 µm in diameter) were seen. APP⁺ elements were present 450 µm from the lesion periphery, but they were most frequent immediately adjacent to the lesion. Three months after initiation of the lesions, the latest time point examined, APP⁺ elements were frequent and distributed around the lesion periphery (Fig. 3B); some of them were seen up to 250 µm from the lesion. It is clear that the number of APP⁺ elements continues to increase throughout the evolution of the DTH lesion, from 1 week to 3 months after its initiation.

BBB in DTH lesions
We investigated whether the time course of axon injury was coincident with the period of BBB breakdown. BBB permeability was assessed in DTH lesions using serial T₁-weighted MRI and a gadolinium-based contrast agent. Signal enhancement around the injection site, indicating breakdown
Fig. 4 Reconstruction of the distribution of APP\(^+\) elements in a representative series of DTH lesions. The shaded area represents the area occupied by the inflammatory infiltrates; the boxed area in the top drawing represents the area analysed. (A) DTH lesions in the cortical white matter. The panels show the change in distribution of APP in lesions from 2 weeks to 3 months after induction. (B) Reconstruction of DTH lesions involving the cortical grey matter overlying the dorsal hippocampus. The panels show the change in distribution from 2 weeks to 2 months.
Fig. 5 T1-weighted MRI images through the centre of DTH lesions. (A) At 7 days, showing a gadolinium-enhanced lesion (arrow) at the site of the DTH lesion. (B) At 31 days, showing a hypointense area (arrow) at the site of the lesion but no gadolinium enhancement.

of the BBB, was observed on images acquired 7 and 11 days but not 3 days after initiation of the lesion. Figure 5(A) shows a typical example of an image showing BBB permeability at day 7. However, in all four animals there was little or no evidence of permeability to contrast agent by 31 days after initiation of the lesion (Fig. 5B), indicating restoration of an intact BBB. At all time points, a focal hypointense region (Fig. 5), thought to be due to the hypercellularity of the lesion, was seen centred on the lesion site in T1-weighted images.

Axon damage caused by MMPs
MMPs have been implicated in the pathogenesis of multiple sclerosis (Opdenakker and Van Damme, 1994). We have shown previously that MMPs 2, 7, 9 and 12 are expressed in DTH lesions and that lesion formation is inhibited by a broad-spectrum MMP inhibitor (Matyszak and Perry, 1996b; Anthony et al., 1998). To investigate whether the MMPs per se can damage axons directly, we microinjected activated MMPs 2, 7 and 9 into the subcortical white matter. APP immunocytochemistry following the injection of MMP-9 into the white matter revealed dramatic evidence of axon injury 24 h after the injection: clearly, large numbers of axons had been severed by the activity of the MMPs (Fig. 3C). MMP-9 produced the most severe axon injury. The injury seen was similar among animals within treatment groups. Vehicle injection alone produced minimal evidence of axon injury (Fig. 6B).

MMPs are known to cause breakdown of the BBB and other tissue damage (Mun-Bryce and Rosenberg, 1998). To investigate whether the action of MMPs was simply non-specific tissue destruction or more specific injury of axons, we injected MMP-9 into the sciatic nerve (Fig. 3D). This resulted in the initiation of marked leucocyte recruitment to the endoneurium; however, APP immunocytochemistry gave only minimal evidence of any axon injury. By contrast, a crush injury to the sciatic nerve (results not shown) led to the generation of significant APP staining. These results demonstrate that CNS axons are highly susceptible to injury by MMPs, which are known to be present in both DTH and multiple sclerosis lesions.

Discussion
In this study, we have demonstrated that the accumulation of macrophages and T cells in a delayed type hypersensitivity reaction within a CNS white matter tract leads to axon injury. The DTH response was directed at BCG antigens, indicating that significant axon damage can occur in the absence of a specific immunological response against axon or myelin antigens. The study further shows that axon injury continues throughout the 3-month period we investigated: it is not restricted to any particular phase of the inflammatory response and in particular it is not restricted to the period of BBB breakdown as detected by gadolinium enhancement in the MRI. We have further shown that MMPs—secretory products of activated macrophages and T cells present in the DTH lesions—have the capacity to cause axon injury and that CNS axons are much more susceptible to this type of injury than are PNS axons. These results suggest that MMPs should be an attractive target for therapeutic intervention to prevent or reduce axon injury in multiple sclerosis.

Detection of axon damage
The current histological method of choice for the investigation of axonal injury is the immunocytochemical detection of APP within axon end-bulbs. APP is transported down the axon by fast axonal transport (Koo et al., 1990). Disruption of this transport mechanism as a result of axonal injury
results in APP accumulating to an immunocytochemically detectable level within axonal end-bulbs, where it is normally undetectable. Closed head injury and spinal cord trauma in man (Aslgren et al., 1996; McKenzie et al., 1996) and traumatic brain injury in rats (Bramlett et al., 1997) are all examples of settings in which APP immunocytochemistry has been used to identify the presence of injured axons. End-bulbs are also detectable by conventional silver staining methods, but in a comparative study immunocytochemical evidence of axonal injury was first detected within 2 h after injury compared with a delay of 15 h when using silver staining techniques (McKenzie et al., 1996). Thus, APP immunocytochemistry permits injured axons to be detected soon after the injury and with considerable sensitivity. It is important, however, to know not only how rapidly the APP end-bulbs are formed but also how rapidly they are cleared.

In the present study, APP end-bulbs in stab lesions were found to appear within 4 h; their number had significantly decreased by 72 h, and this was followed by the disappearance of the vast majority by 1 week after the lesion. This time course of axon end-bulb formation and clearance or resorption is consistent with other studies. Contusion injury to the rat brain results in intense APP immunoreactivity 1–3 days after lesioning, which is followed by the loss of APP end-bulbs by 21 days after the lesion (Lewen et al., 1995). A similar observation was made in a traumatic brain injury model in rats (Bramlett et al., 1997). Although the mechanisms which determine the rate of appearance and disappearance of the APP immunoreactivity remain to be elucidated, the variability in the findings suggests that it may be governed, at least in part, by the brain region in which the lesion occurs. In a study of traumatic brain injury in rats, the accumulation of APP immunoreactivity appeared to show a correlation with the region sustaining the injury (Bramlett et al., 1997); thus, we made a stab lesion in the same region as the DTH lesion and used the same protocol for the APP staining. The evidence presented here shows that the majority of APP end-bulbs are only visible for <=48 h after a cortical stab injury. The presence of large numbers of APP end-bulbs throughout the 3-month period of the DTH lesion strongly suggests that axon injury is ongoing throughout this period.

**BBB and lesion activity**

MRI has made an invaluable contribution to the study of the natural history of multiple sclerosis. A protocol used widely to assess lesion activity is to assay the number of contrast agent enhancing lesions (Kappos et al., 1999) where the BBB is compromised, presumably as a result of the activity of invading leucocytes. In this study we investigated BBB permeability in the DTH lesion using MRI after the administration of contrast agent. We have demonstrated that serial MRI in rodents can be used to follow the evolution of a focal inflammatory response in the rat brain (Blamire et al., 2000). We have shown previously, using horseradish peroxidase as a tracer, that the DTH lesions are associated with BBB breakdown up to 19 days after initiation. In agreement, the current MRI data demonstrate compromise of the BBB between 7 and 11 days after initiation but almost complete restoration by 31 days. Thus, the presence of a lesion with ongoing active axon damage is not necessarily associated with contrast agent enhancement on MRI. Tissue damage continues to evolve behind a closed BBB.

**Mechanisms of axon injury**

It is important to recognize that APP accumulation may represent either transient disruption of anterograde axonal
transport or true transection of the axon. We know that at least some of the injury to the axons, both in multiple sclerosis lesions and in DTH lesions, represents true transection. Using confocal microscopy, Trapp and colleagues demonstrated that a significant proportion of axons in multiple sclerosis lesions were completely severed (Trapp et al., 1998), and axons undergoing Wallerian degeneration have been observed by electron microscopy in the early DTH lesion (Matyszak et al., 1997). The secretory products of activated T cells and macrophages may lead to either arrest of the transport or disruption of axon integrity to the extent that the axon is physically transected.

We have shown that the injection of MMPs into the subcortical white matter leads to the formation of large numbers of APP$^+$ end-bulbs and that axons in this tract are differentially sensitive to certain members of the MMP family. MMP-9 caused more axon injury than MMP-2, and in turn MMP-2 was more damaging than MMP-7. The precise mechanism by which an MMP leads to axon injury is not clear, but the role of extracellular matrix remodelling in neuronal cell death has been demonstrated recently (Chen and Strickland, 1997). MMPs have also been implicated in apoptotic cell death in other organs through modulation of the extracellular matrix (Ishizuya-Oka et al., 2000). Studies using the Wld mutant mouse, in which Wallerian degeneration is delayed for several weeks, show that axon degeneration is an active process akin to programmed cell death (Coleman et al., 1998). Thus, it is possible that MMP degradation of extracellular components in white matter contributes to axon injury. This notion receives some support from our observation that in the PNS, where axons are supported by a robust extracellular matrix and where tissue inhibitors of MMPs (TIMPs) are highly expressed (La Fleur et al., 1996), the axons are less susceptible to MMP-induced degeneration.

Another possible mechanism for the arrest of axonal transport and the eventual transection of the axon is suggested by the work of Smith and colleagues, who have shown that nitric oxide, when present at concentrations found in inflammatory lesions, will lead to degeneration in axons conducting sustained trains of electrical impulses (Redford et al., 1997). Macrophages activated by γ-interferon are known to produce high levels of nitric oxide, which is known to inhibit mitochondrial respiration (Bolanos et al., 1997). The arrest of transport may then lead to breakdown of the axolemma. In addition to MMPs and nitric oxide, activated macrophages secrete TNF-α (tumour necrosis factor α). Although a DTH response is usually associated with the production of inflammatory mediators that produce hyperaemia, we have recently found that the focal intracranial injection of TNF-α produces a decrease in cerebral blood flow rather than the expected increase (N. R. Sibson, P. Styles, V. H. Perry and D. C. Anthony, unpublished results). CNS fibres are sensitive to hypoxia and a hypoxic insult results in the reversal of the Na$^+$–Ca$^{2+}$ exchanger, resulting in an intracellular increase in calcium concentration, which may in turn activate proteases and phospholipases within the axon (Stys et al., 1992).

Apart from the secretory products of macrophages, those of T cells, in particular CD8$^+$ T cells, may also play a role. Recent evidence suggests a correlation between axon injury and the number of CD8$^+$ T cells present in a lesion (Bitsch et al., 2000). CD8$^+$ T cells produce perforin, which, when it is released, results in pore formation in non-specific target cell membranes, resulting in the entry of water, ions and granzymes and subsequent targeted cell death (Murray et al., 1998).

**Absence of immunological specificity**

The presence of damaged axons in a DTH lesion directed against BCG indicates that the damage is not immunologically specific but is a ‘bystander’ effect. The notion of a bystander lesion has a long history and the original studies reported examples of primary demyelination in CNS lesions induced by the immune response to mycobacterium tuberculosis (Wisniewski and Bloom, 1975). Although subsequent studies in the PNS failed to demonstrate primary demyelination, they did report the presence of axons undergoing Wallerian degeneration (Goban et al., 1986; Powell and Hughes, 1987). The potential significance of these observations for immune-mediated multiple sclerosis lesions in the CNS has been overlooked, possibly because axon injury was deemed at that time to be less important, and possibly because the picture in these experimental lesions is greatly complicated by the experimental protocol. The injection of mycobacterium into the neural tissue of an already sensitized animal was made with a large-gauge needle and volumes of antigen were used that could not help but produce significant amounts of tissue damage, independently of the immune response. We have avoided this complication by placing the BCG in the CNS prior to sensitization and allowing any evidence of the tissue damage from the primary inflammatory response to the initial antigen injection to resolve. Thus, any damage produced following the peripheral sensitization is solely a result of the immune-mediated lesion. We have shown in this model that fascicles of fibres surrounding the lesion do undergo primary demyelination (Matyszak et al., 1997), but in addition there is ongoing axon damage mediated by T cells and macrophages throughout 3 months of lesion growth.

**Relevance to multiple sclerosis**

Until recently, demyelination was considered to be the main target in the pathogenesis of multiple sclerosis, with therapies aimed primarily at ameliorating the effects of demyelination or slowing the process. Although the observation that axon injury is a component of multiple sclerosis was first documented nearly 100 years ago, it has been highlighted only recently (Kornek and Lassmann, 1999). Axon injury occurs early in a multiple sclerosis lesion and correlates with the degree of inflammation, showing that it is an integral
part of the pathology (Ferguson et al., 1997; Trapp et al., 1998). We have shown that the presence of activated T cells and macrophages, independently of their antigen target within the brain, will cause damage to axons throughout the evolution of the lesion. The axon damage also continues in the absence of detectable gadolinium enhancing lesions. The number of damaged axons continues to accumulate behind the intact BBB, resulting in an increasingly large and irreversible lesion load, and it is likely that the extent of axon damage may eventually lead to the transition from relapsing–remitting multiple sclerosis to the secondary progressive phase of the disease. Continuing axon injury behind a closed BBB may help to explain why significant reductions in the number of enhancing lesions are not necessarily associated with matched alterations in the long-term progression of the disease. There is a clear need for therapies that will arrest the axon injury as early as possible.

**Acknowledgements**

We thank British Biotech Pharmaceuticals Ltd for providing the purified matrix metalloproteinases. This work was supported by the Multiple Sclerosis Society.

**References**


La Fleur M, Underwood JL, Rappolee DA, Werb Z. Basement membrane and repair of injury to peripheral nerve: defining a potential role for macrophages, matrix metalloproteinases, and


Accepted June 14, 2001.