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THE ROYAL SOCIETY

Axon damage and repair in multiple sclerosis

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It is well known that within long-standing multiple sclerosis (MS) lesions there is axonal loss but whether it is an early or late event has been more difficult to establish. The use of immunocytochemical methods that reveal axonal end-bulbs is a valuable approach to investigating acute axonal injury in human pathological material. The application of these techniques to multiple sclerosis tissue reveals evidence of axonal injury in acute lesions; the distribution of the end-bulbs in acute and active-chronic lesions is associated with regions of maximal density of infiltrating macrophages. Axon injury within the MS lesion will result in both Wallerian degeneration of the axon and also retrograde degeneration of the cell body. The functional consequences of the axon injury will depend upon numbers of axons injured and the topographical organization of the fibres coursing through the lesion.

The molecular mechanisms by which the recruited leucocytes damage or transect the axons are not known. However, investigations in the *Wld* mutant mouse with very slow Wallerian degeneration demonstrate that axon degeneration is not simply a passive disintegration of the axon but has clear parallels with the active processes of programmed cell death. The presence of early axon injury and the consequences of an ever increasing load of neuronal damage has important implications not only for when therapy should be initiated in MS but also the therapeutic target.

Keywords: multiple sclerosis; axon; degeneration; macrophage; *Wld*; Wallerian

1. INTRODUCTION

Multiple sclerosis (MS) is commonly described as a demyelinating disease of the central nervous system and is believed to be an autoimmune disease in which the myelin sheath, or the oligodendrocyte, is targeted by the immune system. The loss of the myelin sheath results in conduction block across this portion of the axon (Smith & McDonald, this issue). The redistribution of ion channels along the demyelinated portion of the axon and the residual capacity of the oligodendrocyte, or its precursor, to remyelinate the axons is consistent with the relapsing–remitting clinical course of the disease in many patients. Within the lesions, or plaques, that are a hallmark of the disease there is an infiltrate of T lymphocytes and macrophages, damage to the blood–brain barrier and myelin loss. There is also, however, clear evidence that in a proportion of lesions there is loss of axons and indeed in some acute aggressive forms of the disease it is accepted that there is severe loss of axons (Charcot 1877; Doinikow 1915; Putnam 1936; Greenfield & King 1936; Lassmann, this issue). The central question to be addressed is not whether there is axonal loss in MS but when and to what extent does the axonal loss occur? The timing and degree of axonal loss is of importance not only in its relationship to the aetiology of the disease but may well be central to the appearance of the clinical symptoms and the progressive deterioration associated with the disease. The fact that axon loss is irreversible has important implications for when, and what type of therapeutic intervention should be used.

2. DETECTING AXONAL DAMAGE

The primary tool available to the neuropathologist for detecting axonal loss in human brain tissue is to stain a tissue section with a method that reveals the neurofilaments within the axons. The more traditional techniques such as the Bielschowsky, and Holmes staining techniques are based on the fact that silver ions bind to neurofilaments with a greater affinity than to other tissue elements (Lowe & Cox 1990). These silver staining techniques are now supplemented by immunocytochemical techniques using antibodies to the different neurofilament components present in the axon (Lowe & Cox 1990). With the silver stains, or immunocytochemistry, it is a simple matter to show that within long-standing MS lesions there is a conspicuous loss of axons (figure 1*a*,*b*). It is also apparent that only a small proportion of the axons are stained and these are the largest diameter fibres.

As is well known, following focal damage to the axon the distal segment separated from the cell body undergoes Wallerian degeneration, and the proximal axon segment connected to the cell body, but now lacking connections with other neurons, dies back. This may eventually lead to the degeneration of the cell body. In some anatomical pathways within the brain, for example the geniculostriate system, the loss of a population of neurons may lead to yet further loss of neurons by retrograde and anterograde transneuronal degeneration (Cowan 1970). It is difficult, if not impossible, to assess from sections stained

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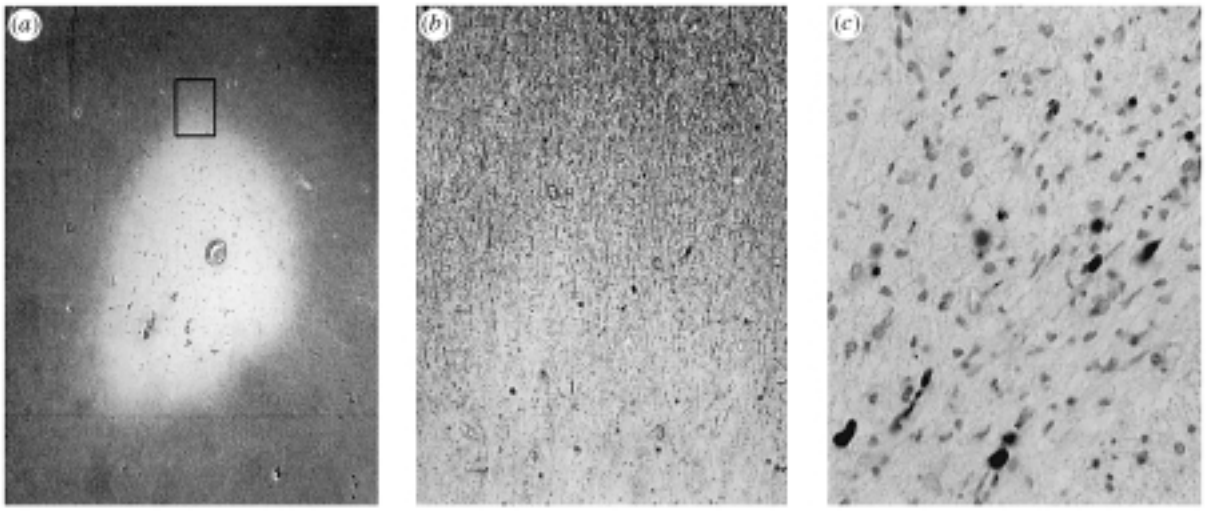

Figure 1. Photomicrographs to illustrate the axon loss in a chronic MS lesion (*a*,*b*). The section has been stained with the Bielschowsky silver stain that reveals the neurofilaments in the larger axons. The region in the box at the border of the lesion in (*a*) is shown at higher power in (*b*). There is a dramatic loss of axons within the lesion. (*c*) A photomicrograph to illustrate the axonal end-bulbs within an acute MS lesion as revealed by immunocytochemistry for amyloid precursor protein.

to reveal the neurofilaments, such as that illustrated in figure 1*a*,*b,* whether the axon was injured during the acute phase of the lesion, or whether the axon degenerated as a consequence of the long-standing demyelination and was then susceptible to another secondary injury from the surrounding inflammatory cells. Another important piece of information that is not available from the examination of a section stained for neurofilaments is which regions of the brain were connected by the axons that have degenerated.

The detection of axon, and axon terminal, degeneration was at one time one of the most important ways in which the connections between different regions of the brain were worked out. A focal lesion to a brain region gave rise to axon degeneration and the degenerating axons could then be traced to their targets by taking advantage of the greater affinity of dying axons for silver relative to intact axons. The application of the sensitive and selective methodology offered by the Nauta method and its modifications (Nauta & Gygax 1954; Fink & Heimer 1967) led to a revolution in our understanding of brain circuitry. However, these methods are not readily applied to human post-mortem material since they require strict fixation routines, are sensitive to both the survival time after the lesion and the post-mortem interval, and require experienced laboratory staff to carry out the staining protocols. The experienced application of these methodologies is a dying craft although there have been some recent modifications for use with human post-mortem material (Miklossy & Van der Loos 1990).

Electron microscopy, which is routinely used in the laboratory for the detection of dying axons, or evidence of axon loss, is also difficult to use on human material. The fixation is a matter of considerable importance and the preservation of the tissue is rarely adequate except in some biopsy specimens. Even if it is possible to detect dying axons, the quantification of axon loss by electron microscopy poses considerable difficulty. The individual variation in neuron numbers in the human central nervous system (CNS), even in well-defined structures (Curcio & Allen 1990) means that samples from a large number of individuals, both normal and diseased, would have to be examined before any meaningful quantitative values could be assigned.

In short, the detection of axon damage in human post-mortem material is not straightforward, its presence in MS and indeed other neurodegenerative diseases is poorly documented and thus its significance is likely to have been greatly underestimated.

3. AXON DAMAGE IN EARLY MULTIPLE SCLEROSIS LESIONS

Although there are reports that axon damage is present in MS lesions these observations have in general been neglected when compared with the striking and readily observed loss of the myelin sheath with the relative preservation of the axons. The emphasis of the MS lesion as an essentially demyelinating lesion has received considerable support from studies on experimental allergic encephalomyelitis where the clinical signs, damage to the blood–brain barrier and loss of myelin have been emphasized. Despite clear evidence from Guillain–Barré syndrome, a disease of the peripheral nervous system sharing numerous pathological features with MS, that axonal damage is common (Hartung *et al.* 1996) the loss of axons in MS has not until recently been a focus of interest. The clinical and neuropathological picture from studies of Guillain–Barré syndrome show that axons can be acutely damaged by a cellular and humoral immune-mediated assault against peripheral nervous system antigens.

Evidence that significant axon damage occurs early in MS lesions has come from two different approaches; the application of new neuropathological techniques to post-mortem MS tissue and the application of magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) in the living patient. It has long been known that following transection of an axon the proximal end of the axon rapidly seals and the cut end

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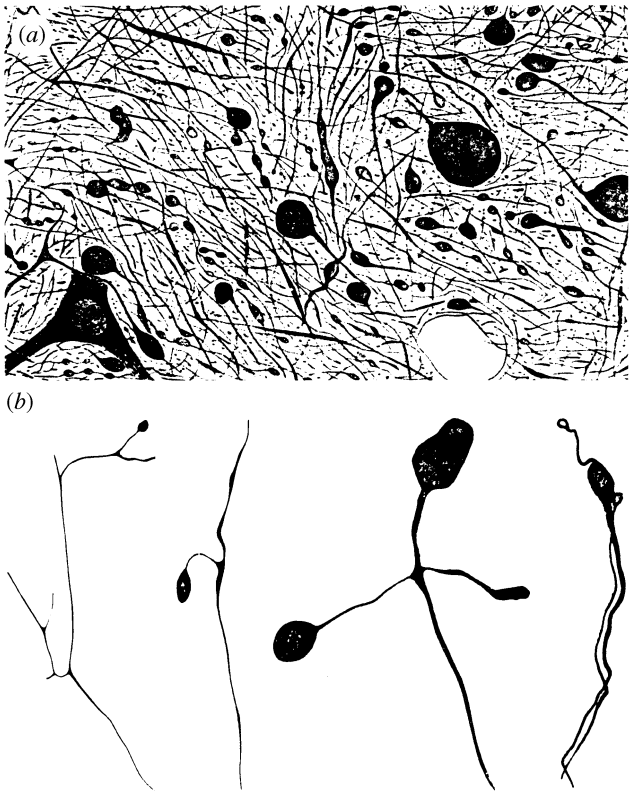

Figure 2. The figures are taken from Doinikow (1915). In (*a*) the appearance of numerous axonal end-bulbs in an MS lesion is revealed by the Bielschowsky silver stain. In (*b*) a number of profiles are illustrated that suggest that the damaged axons in an MS lesion are attempting to regenerate.

swells to form an axon end-bulb (Cajal 1928). The swollen end-bulb represents the build-up of proteins being transported to the cut end of the axon by anterograde axonal transport. Sherriff *et al*. (1994) took advantage of this phenomenon to show that a number of neuron-associated proteins, transported by rapid anterograde axonal transport, could be detected in the axon end-bulb but these same proteins could not be detected in normal axons. Thus, tissue sections stained with antibodies against the amyloid precursor protein (APP) could be used to detect axon end-bulbs in the white matter of persons who had died from acute head injury. The application of this technique to models of acute axon damage and to human post-mortem tissue has gained wide acceptance as a sensitive and valuable methodology for the detection of acute axon damage. We used this technique on samples of tissue taken from persons who had died with MS (Ferguson *et al*. 1997).

The application of APP immunocytochemistry to MS lesions of varying stages revealed that numerous APP-positive end-bulbs were a consistent feature of acute and acute–chronic lesions (figure 1*c*). The large number of end-bulbs in the most acute lesions, and their virtual absence in chronic lesions, led us to estimate the distribution of end-bulbs and macrophages in acute, acute–chronic and chronic lesions. We found a clear relationship between the numbers of end-bulbs and the numbers of macrophages, the maximum numbers of APP-positive end-bulbs being present in the regions of maximal macrophage density (Ferguson *et al*. 1997). These observations suggested that it is the ongoing inflammatory response that is responsible for the damage to the axons. The appearance of the swollen axon end-bulbs is similar to the appearance of damaged axons illustrated by Doinikow (1915) (figure 2*a*).

One possible problem with the interpretation of the APP-positive elements seen in these lesions is that they may represent a temporary arrest of axoplasmic transport rather than a transection of the lesion. The study of Trapp *et al*. (1998) also indicates that axon damage happens early in the lesion and that complete axon transection occurs. Using an antibody that detects non-phosphorylated neurofilaments in demyelinated axons and confocal microscopy they demonstrated the presence of axon end-bulbs in acute MS lesions and that these end-bulbs were only attached at one point.

In both of these studies it was apparent that there were large numbers of damaged axons in acute lesions. However, at the present time it is still not possible to give a good estimate of just how many axons are damaged and whether the damage is biased to axons of a particular size. From a quantitiative perspective the significance of each axon end-bulb will in part depend on the time for which it is visible. If the end-bulb stained by APP, or any other method, is a short-lived structure but can be found in all active MS lesions then even a small number of these elements will be important. A useful analogy can be drawn here with the short-lived appearance of apoptotic cells. The apoptotic nucleus is only visible for a few hours and its rapid clearance led to the significance of programmed cell death being greatly undervalued until the last decade or so (Raff 1992).

The other significant technical advance that has drawn attention to axon loss in MS is the use of MRI and MRS. The application of techniques to quantify images from MRI has revealed that there is significant tissue atrophy in the spinal cord and brain in patients with MS (Losseff *et al*. 1996*a*,*b*; Smith & McDonald, this issue). These measures of whole tissue cross-sections, or volume, do not, however, discriminate between myelin and axon loss. A powerful technique for the analysis of the biochemical components of tissue in life, is MRS. The normal proton spectrum in brain tissue is dominated by a signal from N-acetyl aspartate (NAA), an amino acid that appears to be largely if not wholly localized to neurons, their cell bodies and processes including the axon (Urenjak *et al*. 1993). There are a number of studies showing that NAA is decreased in MS lesions but importantly also in apparently normal white matter (Davie *et al*. 1994, 1997; Fu *et al*. 1998). The loss of the NAA from white matter distant from an identifiable MS lesion is not surprising given that the distal segment of a transected axon will undergo Wallerian degeneration and the proximal segment will die back. It is worth pointing out that in some studies it has been reported that as the inflammatory lesion resolves during a period of remission the NAA signal is restored to normal levels (Davie *et al*. 1994; De Stephano *et al*. 1997). Although this may represent some recovery of axons from reversible axon damage, it may equally represent the resolution of the oedema and as the axon packing is restored to normal levels so the NAA signal increases. It is not possible to explain the loss of NAA distal to a lesion by reference to the local oedema but it is not possible to

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rule out distal effects from inflammatory cytokines generated within the lesions that may influence neuronal NAA. The loss of NAA and its relationship to disability is considered in further detail in Smith & McDonald (this issue).

4. MECHANISMS OF AXON DAMAGE

The recent studies described above demonstrating axon damage in early MS lesions clearly implicate inflammatory cells as being responsible for the damage. What might be the mechanisms by which the combined activities of T lymphocytes and macrophages lead to the transection of an axon? Although axon damage in Guillain–Barré syndrome has been documented and almost certainly underlies the residual permanent disability there has been little research directed at specific mechanisms. In experimental allergic neuritis, which mimics many aspects of the human peripheral nerve inflammatory pathology (Hartung *et al.* 1996), it is clear that the degree of axon damage is related to the intensity of the inflammatory response. The greater the initial challenge the greater the inflammatory response within a peripheral nerve and the damage to both myelin and axons. The axon damage is described as 'bystander damage', implying that there is no immunological specificity, but gives little idea of the molecular mechanisms involved. It is of interest to consider whether other models of axon injury can provide any insights into the processes of axon degeneration.

The most widely used model to study the mechanisms of axon degeneration is the Wallerian degeneration following axon transection. Despite the apparent simplicity of this system the sequence of molecular events resulting in nerve degeneration are poorly understood. Within hours of injury to a peripheral nerve the mitochondria and other organelles accumulate in the nodal and paranodal regions of the isolated axon stump. At this stage fast axonal transport continues at normal rates and electrical conduction and synaptic activation can be elicited. The axonal cytoskeleton abruptly breaks down with the apparently simultaneous disintegration of the axonal organelles, axolemma and axonal cytoskeleton (George & Griffin 1994). The period of electrical excitability may last for at least a day in mouse sciatic nerve and for two to three days in mouse optic nerve, with the larger fibres degenerating more rapidly than the smaller ones (Lunn *et al.* 1989). It is not known what determines the different survival rates of axons from the peripheral and CNS. It seems likely that calcium influx into the nerve activates calcium-activated proteases, such as the calpains (Nixon *et al.* 1986) and these proteases are involved in the degradation of the cytoskeleton of the axon and it loses its integrity. Recent studies from the *Wld* mouse, a spontaneously occurring mutant, show that axon degeneration is initiated by an active process rather than lack of maintenance from the cell body.

The *Wld* mutant mouse (Coleman *et al.* 1998) has provided novel insights into the process of axonal degeneration. In contrast to the rapid degeneration of the distal segment that follows transection of a peripheral or central axon in all mammals the distal segments of cut axons in the *Wld* mouse strain have the capacity to survive for two weeks or more following transection (Lunn *et al.* 1989; Perry *et al.* 1991). The axons in the nerve segment appear morphologically normal and when stimulated electrically will conduct an action potential. The slow Wallerian degeneration phenotype is maintained *in vitro* and is a property of isolated neurons (Buckmaster *et al.* 1995). These simple observations indicate that in the wild-type nerve there must be mechanisms that produce the rapid destruction of the distal segment of the axon. The parallels with the active auto-destructive events in programmed cell death, which appear as apoptosis of the cell body, are obvious. Further evidence of the similarities between Wallerian degeneration and programmed cell death have come from studies on processes cut off from the neuronal cell body *in vitro* (Buckmaster *et al.* 1995). In these studies it was demonstrated that manipulations which slow apoptosis of the cell body of neurons also slow the degeneration of axons. Scanning electron microscopy reveals that the early stages of the degeneration of the neurites involve the formation of blebs at the axolemma similar to the blebbing seen on the surface of many cell types undergoing apoptosis. The evidence that there exist in axons mechanisms that actively initiate or suppress the degeneration of the axon is intriguing.

One of the consequences of traumatic head injury is the widespread axon damage in the white matter (Gentleman *et al.* 1995). Until recently the widely held view was that the axons were sheared, or literally torn apart, by the forces exerted on the axon during the injury. This view has been revised since it has been demonstrated that there were rather few axons sheared by the initial injury (Povlishock & Jenkins 1995). The picture that emerges from recent studies is that within a few hours of the lesion there is a local arrest of axoplasmic transport, which is associated with local disruption of the microtubules, and in more severe injury compaction of the neurofilaments with loss of their side-arms (Povlishock & Jenkins 1995). This local loss of axonal transport eventually results in local breakdown of the axolemma and the formation of an end-bulb. These studies, however, beg the question as to what is responsible for the local arrest of axonal transport. It has been shown that there are focal alterations in the permeability of the axolemma, since segments of the axon become permeable to horseradish peroxidase, but it is not known what endogenous molecules enter the axon. The authors have suggested that calcium is unlikely to be the key molecule since the morphological appearance appears to be distinct from the early granular disintegration of the cytoskeleton which is seen during Wallerian degeneration. The key molecules that cross the axolemma and cause arrest of axon transport remain to be elucidated.

Another mode of axon injury that has come under investigation is the effect of hypoxia, since white matter damage is obviously a very important component of an ischaemic insult. The white matter of the CNS is very sensitive to hypoxia and indeed more so than peripheral nerves (Stys *et al.* 1995), an interesting contrast to the differential rates of Wallerian degeneration described above where the degeneration is more rapid in the peripheral nervous system. Stys *et al.* (1992) have studied the effects of hypoxia on the rat optic nerve as a model of a CNS fibre tract. They have shown that following a hypoxic insult the Na^+–K^+ ATPase is compromised and

the level of intracellular Na^+ rises, this is in turn accompanied by a reversal of the Na^+–Ca^{2+} exchanger resulting in a net rise in intracellular Ca^{2+}. This rise in intracellular calcium would then activate axoplasmic phospholipases and proteases resulting in the demise of the axon. It is unclear, however, whether this process is relevant to MS lesions, given that there is a local inflammatory process in the MS plaque and that inflammation is usually associated with an increase in blood flow rather than hypoxia. Despite this it remains an important issue to establish whether blood flow in the MS lesion is normal, and whether the lesions are or are not hypoxic.

There are a number of animal models that reproduce aspects of MS. The pathology of the most widely studied model, experimental allergic encephalomyelitis, is dominated by oedema and demyelination, although there have been reports of axon damage in both the acute and chronic forms of the disease (Raine *et al.* 1980; Raine & Cross 1989). A useful model to study immune-mediated mechanisms of axon injury is the bystander demyelination model (Matyszak & Perry 1995). In this model a delayed-type hypersensitivity (DTH) reaction to bacillus Calmette–Guérin (BCG) deposited within the brain parenchyma leads to infiltration of leucocytes, blood–brain barrier breakdown, and myelin and axon damage. One particular advantage of this model is that the lesion appears at a known time and location within the brain with a high degree of reproducibility. From this model it is clear that axon injury appears very rapidly at the lesion site and that axon injury is plentiful (Matyszak *et al.* 1997). There is no requirement for an immune component directed against a CNS antigen. Activated macrophages secrete a plethora of molecules, a number of which have the capacity to damage the myelin sheath and axons including matrix degrading metalloproteinases (Anthony *et al.* 1998).

Although it is unclear whether demyelination is a prerequisite for axon injury in the MS lesion, the very rapid appearance of injured axons in the DTH lesions makes it likely that axon injury may take place at the same time or even precede myelin injury. It is worth noting, however, that demyelinated axons may be more susceptible than myelinated axons to secretory products of leucocytes. It has been demonstrated that nitric oxide, a secretory product of activated macrophages, can induce reversible conduction block in demyelinated axons (Redford *et al.* 1997), and when nitric oxide is combined with sustained axonal activity can induce axon degeneration (see Smith & McDonald, this issue).

5. EVIDENCE OF REGENERATION AND REPAIR

Axon injury in the CNS is of particular importance because of the essentially irreversible nature of the lesion. Considerable advances have been made in defining the factors that inhibit regeneration in the CNS, including components of myelin (Schwab 1996) and extracellular matrix proteins (Fitch & Silver 1997) and also the factors that promote regeneration including the neurotrophins (Aguayo *et al.* 1996). However, the induction of significant functional repair in the brain remains a major challenge and, in the context of repair of lesions scattered throughout the cerebral white matter, a truly daunting task. Indeed it should also be remembered that despite the rapid and effective regeneration that takes place in the peripheral nervous system of small mammals, effective regeneration of major peripheral nerves in man is often associated with problems as well as benefits.

It is of interest in the context of the MS lesion to ask whether this inflammatory reaction which leads to axon damage is accompanied by evidence of repair processes. Within the lesions it is not only the axon that is damaged but also the blood–brain barrier and the myelin sheath. It is clear that the blood–brain barrier is repaired or restored in lesions, during remission. The mechanisms underlying the rapidly reversible nature of blood–brain barrier breakdown are not well defined. The oligodendrocyte has some capacity to remyelinate the demyelinated axons. There is much interest in trying to discover the rules that regulate this remyelination process and whether de-differentiated oligodendrocytes or oligodendrocyte precursors can be encouraged to engage in this process more effectively (Scolding, this issue). Although axon damage in MS lesions has been known about for a long time (see Lassmann, this issue) there are remarkably few data available on the attempts that axons may make to regenerate in these lesions. Doinikow (1915) illustrates axons from lesions that are clearly generating abortive sprouts (figure 2*b*) but it is unclear how common this feature is and at what stage of lesion development this takes place. There is much to be done in this regard.

6. CONSEQUENCES OF AXON INJURY

A striking feature of a coronal section through the human brain, when compared with a section through the brain of commonly studied laboratory species such as rat or monkey, is the shear size of the cortical fibre tracts when compared with the thickness of the cortical grey matter. If lesions, be they local infarcts or MS plaques, were to be scattered at random in the cortical grey and white matter of man, a large proportion would invariably lie in the white matter. The functional impact of an MS lesion in a cortical white matter tract will not only depend on the location of the lesion but will also critically depend on the precision of the fibre topography at that location. If the fibres connecting one region of the cortex to another brain structure run in a tightly organized bundle then a relatively restricted lesion that damages only a small proportion of the axons in this tract might be expected to produce a functionally measurable impairment. If, on the other hand, there is little precision in the fibre topography and the fibres from different regions are intermingled, then the evolving lesion will not only have to be larger to produce the same functional impairment but would take longer to reach a critical number of damaged axons before an impairment appears.

There has been considerable interest in the degree of fibre topography in different fibre tracts of the brain and the potential role that this may play in the guidance of axons to their targets and their interactions with other fibre populations (Horton *et al.* 1979; Molnar & Blakemore 1995). One region of the brain that has been barely studied, however, is the order of fibres in the cortical white matter and little is known of this. Furthermore, it is clear that for the study of cortical white matter these

studies should ideally be carried out in the primate brain. The application of the appropriate staining techniques to post-mortem human material with localized lesions (Miklossy & Van der Loos 1990; Burgel *et al.* 1997) is likely to give some idea of the degree of topographical precision of cortical fibre tracts in man.

The transection of axons within the MS lesion will not only lead to Wallerian degeneration of the distal stump but may also lead to loss of the neuronal cell body, and even possible further anterograde and retrograde degeneration as described above. The burden of the lesion may be further increased by the secondary degeneration of oligodendrocytes and additional demyelination of intact fibres. Following injury to the spinal cord and damage to spinal fibre tracts there is Wallerian degeneration of axons rostral and caudal to the lesion. Shuman *et al.* (1997) have shown that along these tracts of degenerating fibres there are oligodendrocytes undergoing apoptosis. The loss of oligodendrocytes from regions of the spinal cord, distant to the primary lesion, accounts for the demyelination distal to the primary lesion. This demyelination may lead to conduction block and further functional impairment. Oligodendrocytes normally myelinate segments of several different axons, each of which provides some trophic support to the oligodendrocyte. The authors argue that when a proportion of the axons myelinated by a particular oligodendrocyte undergo Wallerian degeneration, the oligodendrocytes undergo apoptosis and degenerate because they have lost a critical amount of trophic support. The intact axons, that were insufficient to maintain the oligodendrocyte, now have a demyelinated segment. This demyelination would be expected to contribute further to the functional deficit. If this secondary degeneration of oligodendrocytes occurs following axon damage after spinal cord injury we might expect to find oligodendrocytes undergoing apoptosis at some distance from an MS lesion. This form of degeneration would be particularly conspicuous in regions where the fibre topography is more ordered such as in the spinal cord or optic nerve.

7. CONCLUSION

There is a growing body of evidence to indicate that in acute MS lesions there is ongoing axon injury. The degree of axonal injury appears to reflect the intensity of the inflammatory response. It is likely that the cumulative loss of axons contributes to the irreversible functional disability that is present in many patients. There is much work to be done to evaluate the variability of axon loss in different stages of lesion evolution and its contribution to disability in different stages or different forms of MS. A better understanding of the organization of fibre order in the cortical white matter will help in the interpretation of lesion impact and will also be of value in other pathologies where white matter lesions are common, for example stroke and trauma.

Although it is clear that inflammatory cells are involved in damaging the axons, the molecular mechanisms of axon damage are poorly understood. Recent evidence shows that axon degeneration following injury has similarities with the cellular mechanisms underlying programmed cell death. A primary goal must be to understand the events that trigger axon degeneration since this will lead to novel ways of preventing this irreversible lesion and possibly arresting disease progression.

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