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SYMPOSIUM: Oxidative Stress in Neurological Disease

Demyelination: The Role of Reactive Oxygen and Nitrogen Species

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This review summarises the role that reactive oxygen and nitrogen species play in demyelination, such as that occurring in the inflammatory demyelinating disorders multiple sclerosis and Guillain-Barré syndrome. The concentrations of reactive oxygen and nitrogen species (e.g. superoxide, nitric oxide and peroxynitrite) can increase dramatically under conditions such as inflammation, and this can overwhelm the inherent antioxidant defences within lesions. Such oxidative and/or nitrative stress can damage the lipids, proteins and nucleic acids of cells and mitochondria, potentially causing cell death. Oligodendrocytes are more sensitive to oxidative and nitrative stress *in vitro* than are astrocytes and microglia, seemingly due to a diminished capacity for antioxidant defence, and the presence of raised risk factors, including a high iron content. Oxidative and nitrative stress might therefore result *in vivo* in selective oligodendrocyte death, and thereby demyelination. The reactive species may also damage the myelin sheath, promoting its attack by macrophages. Damage can occur directly by lipid peroxidation, and indirectly by the activation of proteases and phospholipase A_2. Evidence for the existence of oxidative and nitrative stress within inflammatory demyelinating lesions includes the presence of both lipid and protein peroxides, and nitrotyrosine (a marker for peroxynitrite formation). The neurological deficit resulting from experimental autoimmune demyelinating disease has generally been reduced by trial therapies intended to diminish the concentration of reactive oxygen species. However, therapies aimed at diminishing reactive nitrogen species have had a more variable outcome, sometimes exacerbating disease.

Recent years have witnessed a burgeoning of interest in the role that reactive oxygen and nitrogen species (ROS and RNS) play in inflammation, and also in the role that inflammation plays in multiple sclerosis (MS). These interests have naturally focused attention on the potential role of ROS and RNS in demyelinating disease, the subject of this review.

Oxidative And Nitrative Stress

Cells within the nervous system are routinely exposed to low concentrations of potentially deleterious reactive oxygen and nitrogen species, but these normally pose little threat since cells possess an arsenal of defence and repair mechanisms. However, events such as inflammation can conspire to increase the production of these reactive species dramatically, and this may overwhelm the cell's defences resulting in a condition known as oxidative and/or nitrative "stress". Such stress may lead to changes in the properties of the cell's constituent molecules, notably the lipids, proteins and nucleic acids. The ROS and RNS of primary concern are the superoxide anion ($O2^{\bullet -}$, hereafter called superoxide), nitric oxide (nitrogen monoxide, $^{\bullet}NO$)♣, peroxynitrite ($ONOO^-$; a product of the combination of superoxide and nitric oxide), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$). Other potentially important species include singlet oxygen ($^1O_2$), nitrogen dioxide radical ($NO_2^{\bullet}$), nitrosonium (NO^+) and nitronium (NO_2^+)

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♣ The dot signifies that the compound is a radical, namely a compound possessing one or more unpaired electrons, i.e. electrons that occupy an atomic or molecular orbit by themsleves. This configuration can make the compound reactive. Superoxide and nitric oxide are both radicals, but they are not as reactive as some of their derivatives.

ions, the perhydroxyl radical ($HO_2^{\bullet}$), and hypochlorous acid (HOCl), but since there is little direct evidence so far that these species play an important role in demyelination, they will not be discussed in this review. The reactions initiated by the reactive species include peroxidation of lipids, nitrosylation of thiol groups, nitration of tyrosine, and oxidation and deamination of nucleic acids. Reactions such as these may directly, or indirectly, result in demyelination. If this is correct, then novel therapies for demyelinating disorders may arise from the introduction of strategies aimed at modifying the production and fate of ROS and RNS. This review describes the evidence that ROS and RNS play a role in demyelination, the mechanisms believed to be involved, and observations made from trial therapies in demyelinating disorders.

Inherent Vulnerability Of The Nervous System To Oxidative Damage

The CNS is inherently vulnerable to damage mediated by ROS. First, it is very active in oxidative metabolism. Such activity results in a relatively high intracellular production of superoxide, since 2-5% of the oxygen consumed in mitochondrial electron transport is converted to superoxide. Superoxide is converted by the enzyme superoxide dismutase (SOD) to hydrogen peroxide and oxygen (98). Hydrogen peroxide is normally reduced to water by the action of catalase or glutathione peroxidase (with reduced glutathione as co-substrate), but in the presence of decompartmentalised transition metals such as iron and copper it can be converted to the highly toxic hydroxyl radical (98). This radical is so reactive that it has a diffusion radius of only approximately 0.3 nm before it interacts with another molecule (11). For comparison, the interlamellar spacing of myelin is approximately 10 nm. Second, the CNS has a very limited ability to conduct anaerobic glycolysis, and so it is unusually vulnerable to hypoxia. This is a concern since the production of superoxide by mitochondria increases dramatically in low oxygen concentrations (reviewed in (10)). Third, some cells in the CNS, notably oligodendrocytes, have relatively low levels of antioxidant defences combined with a high iron content and extensive elaborations of membranes. These features all predispose oligodendrocytes, in particular, to oxidative damage. Finally, myelin membrane is a preferential target of ROS (24) due to its composition and high lipid to protein ratio.

It has been proposed that the brain may be much more vulnerable to oxidative stress than the spinal cord or peripheral nerve (139). However, the levels of the antioxidant glutathione and glutathione related enzyme activities appear to be lower in peripheral nervous tissue than in the central nervous system, suggesting that this protective mechanism, at least, is less effective in the peripheral nervous system (188).

Cellular Oxidative Defence Mechanisms

Cells employ a range of defence mechanisms to protect themselves from oxidative and nitrative injury. The strategy includes mechanisms to limit the production of reactive species, to scavenge any radicals that are produced, to prevent the proliferation of secondary radicals in chain reactions, and to repair the damage that may nevertheless occur. This strategy is achieved by the presence of antioxidant enzymes such as SOD, glutathione peroxidase and catalase, and low molecular weight antioxidants such as glutathione and dietary vitamins C and E. There are two forms of SOD. Mn-SOD is located in mitochondria, and Cu/Zn-SOD is located within the cytosol. Both isoforms specifically catalyse the dismutation of superoxide to hydrogen peroxide and oxygen, thereby protecting cells from the formation of the more harmful peroxynitrite. Glutathione peroxidase is an important cytosolic selenium-containing enzyme which converts peroxides, notably hydrogen peroxide and lipid peroxides, to water and oxygen. The enzyme requires reduced glutathione as an electron donor. Catalase converts hydrogen peroxide in peroxisomes to water and oxygen. Glutathione is pivotal to oxidative defence and performs the non-specific reduction of many ROS and RNS. It can also reactivate some enzymes which have been inhibited by exposure to oxidants. Glutathione is found at high concentrations in cells, particularly in astrocytes (~5 mM) (230). Vitamin C (ascorbate) is another important, non-specific antioxidant, which accumulates in the CNS particularly in astrocytes (up to 10 mM) (208, 209). Vitamin C can regenerate glutathione and vitamin E from their radicals *in vitro*, returning them to the antioxidant pool. Vitamin E (α-tocopherol) is a lipid soluble antioxidant, and forms a primary defence against lipid peroxidation. It can break the chain reactions of lipid peroxidation by reducing peroxyl radicals. The chemical biology of ROS and RNS has been reviewed in detail elsewhere (98, 186, 249).

Other recent reviews of potential interest examine the neurobiology of $^{\bullet}NO$ (259), the role of $^{\bullet}NO$ in mitochondrial damage (19), the effects of oxidative stress on astrocytes (173), the role of astrocytes in antioxidant defence (245), and the role of microglia in brain damage (151).

The Role Of Nitric Oxide In Demyelination

•NO has important physiological roles in the regulation of vascular tone, in intra- and inter-cellular communication, and in the destruction of microbes and tumour cells (for review see (226)). It is an important inflammatory mediator which also appears to exert significant effects in inflammatory demyelinating diseases.

Elementary Chemistry of Nitric Oxide. •NO is produced from L-arginine by a family of homodimeric enzymes, termed nitric oxide synthases (NOS), which require several co-substrates and co-factors, including oxygen, and NADPH. The family is made up of three members, nNOS (for "neuronal" NOS; also termed type I NOS), eNOS (for "endothelial"; also termed type III) and iNOS (for "immunological", "inflammatory" or, most commonly, "inducible"; also termed type II) which are the products of separate genes on different chromosomes. Two forms, eNOS and nNOS, are expressed constitutively in endothelial cells and in some neurons respectively, and have collectively been termed cNOS. The inducible form, iNOS, differs from cNOS in two important ways. First, it produces relatively large amounts of •NO compared to cNOS, and second, it is effectively independent of Ca^{++} (for review see (151)).

•NO itself is not thought to be highly toxic at the concentrations formed *in vivo*. However •NO reacts rapidly with some molecules, particularly transition metals and other free radicals, and these reactions can lead to the formation of more damaging species (12). •NO has a short half-life (~1 second) in tissues. The chemistry of •NO and its derivatives is complex (reviewed in (11, 12, 14, 59)). Notably, •NO combines with superoxide to form the potent oxidising and nitrating agent, peroxynitrite. The rate constant for this reaction is sufficiently high that •NO out-competes SOD for superoxide (110). However, the relatively high concentration of SOD in the cell usually means that intracellular superoxide concentrations are kept sufficiently low that peroxynitrite formation is normally minimal. Under some conditions, however, such as during the oxidative burst, excess superoxide is produced and then extracellular peroxynitrite formation can be substantial (for review see (12)). Peroxynitrite is very reactive, and has a short half-life (milliseconds) in tissues. There is evidence that reaction of peroxynitrite in the presence of carbon dioxide enhances its nitrating properties. In fact, it is now believed that many of the deleterious effects ascribed to •NO may in reality be due to peroxynitrite and the agents derived from it (including peroxynitrous acid, hydroxyl radicals and nitrogen dioxide radicals, and the nitronium ion). Therefore, reference to "•NO" in this review should not be taken to exclude its metabolic derivatives (i.e. peroxynitrite etc.).

The Production Of Nitric Oxide In The Nervous System In Response To Inflammation. The induction of iNOS, or the production of substantial amounts of •NO, has been reported in astrocytes (102, 206), microglia (18, 47, 264), a subset of oligodendrocytes (148), Schwann cells (82), and cerebral endothelium (26, 202). In addition, iNOS induction has been widely reported in macrophages and other cells which can invade the nervous system and participate in inflammatory reactions. Typically, the induction of iNOS is achieved experimentally by stimulation of cells with bacterial cell wall lipopolysaccharide (LPS), and/or the use of pro-inflammatory cytokines such as interferon-γ (IFNγ). However, CSF from 34% (13/38) of MS patients has been found to promote •NO production in mixed cultures of rat oligodendrocytes, microglia and astrocytes, vs. only 10% (2/20) of CSF samples from controls. Increasing •NO concentrations were accompanied by a proportional reduction in the number of viable oligodendrocytes (253).

Much of the work on the stimulation of iNOS expression has been carried out on rodent macrophages, but species differences can be prominent. While cultured human microglia have sometimes been reported to express iNOS upon LPS stimulation (42), other studies have not observed this effect (44, 43, 138). Human macrophages are, however, capable of producing significant amounts of •NO in response to certain stimuli, including exposure to certain protozoans (203). There are also likely to be species differences in the control of iNOS expression (for review see (69)), suggesting that particular caution is needed when extrapolating rodent data to humans. Human astrocytes, in contrast with macrophages, are capable of producing substantial •NO in response to stimulation by proinflammatory cytokines (see below)(108, 138), although iNOS expression is not affected by LPS exposure (138).

Control of Nitric Oxide Production. Most studies in this field suggest that the control of iNOS expression occurs predominantly at the transcriptional level and involves a number of transcription factors, including nuclear factor kappa-B (for review see (151)). Differences in transcription factor expression and the differential presence of consensus sequences are likely to be related to observed differences in species and tissue expression of iNOS. Post-transcriptional control of iNOS also occurs. For example, in mouse macrophages,

transforming growth factor-β (TGF-β) reduces iNOS activity by destabilising iNOS mRNA, reducing translation and increasing the degradation of iNOS (238).

The up-regulation of •NO production in glia using LPS, or LPS+IFNγ, has been shown to involve mitogen-activated protein (MAP) kinase cascades. Specific inhibition of either of two MAP kinases (extracellular signal-regulated kinase (ERK) or p38 kinase) reduces both the expression of iNOS and the production of •NO in primary cultures of rat glia, while inhibition of both kinases leads to almost complete inhibition (13).

The immune response encompasses a number of chemical interactions which regulate •NO production. For example, there is evidence that some eicosanoids can down-regulate •NO production. Addition of exogenous prostaglandin E_2 (PGE_2) to rat microglial cells down-regulates iNOS by increasing cAMP levels (152), and inhibition of cyclooxygenase increases •NO production in activated human astrocytes (116). Paradoxically, however, inhibition of cyclooxygenase in rat microglia decreases iNOS expression (152). These findings indicate that products of the arachidonic acid cascade may have both positive and negative regulatory effects on •NO production. •NO also participates in its own regulation. In activated astrocytes, the addition of •NO -trapping agents increases the transcription of iNOS, indicating that •NO provides direct negative feedback on the enzyme responsible for its generation (170). An important "sink" for the removal of •NO is the vasculature, since •NO readily diffuses through tissue and reacts with oxyhaemoglobin to form methaemoglobin.

Cytokines also have a significant effect on iNOS expression and •NO production. In general, the pro-inflammatory cytokines such as interleukin-1α (IL-1α) (236), IL-1β (138, 207), IFNγ (207) and tumour necrosis factor-α (TNFα) (155) induce, or increase, the production of •NO in glia. The cytokines can act synergistically: for example, neither IFNγ nor TNFα cause significant •NO production by cultured astrocytes, but the combination of cytokines elicits a strong response (223). Glia can themselves synthesize cytokines which in turn lead to further up-regulation of iNOS. For example, the release of TNFα from astrocytes can cause the expression of iNOS in the cerebral endothelium (202). There is also recent evidence that TNFα and •NO may mutually provoke the production of the other *in vitro* (223). Anti-inflammatory cytokines such as IL-4 (207), IL-10 (207) and TGF-β1 (TGF-β1) (237) decrease the production of •NO in activated rodent glia in culture. Reports of similar suppression in human astrocytes vary. Liu *et al*. (138) found no inhibition of iNOS or •NO production, while Hu *et al* (108) found that all three cytokines acted as potent inhibitors of pro-inflammatory cytokine-induced •NO production in cultured human fetal astrocytes. IFNβ, which primarily exhibits anti-inflammatory properties, reduces •NO production in an astrocytoma cell line (90) and in primary astrocyte cultures (109). In some cases, the inhibitory action of these cytokines is mediated by blocking the production of pro-inflammatory cytokines (168). Thus the control of iNOS by cytokines involves both positive and negative interactions.

•NO production by phagocytes can be affected by exposure to myelin. Phagocytosis of rat myelin by rat macrophages *in vitro* leads to an increased production of •NO, and this effect can be enhanced by opsonizing the myelin with anti-myelin antibodies (157). The effect of phagocytosis is not limited to myelin, since activated microglia also produce more •NO after phagocytosing certain bacteria and latex beads (53).

There is also evidence that cells can modulate their expression of their oxidative and nitrative defence mechanisms, either in response to the challenge of oxidative and nitrative stress, or even in anticipation of such challenge. For example, cultured microglial cells respond to a LPS challenge by the induction of enzymes which produce high, antimicrobial concentrations of •NO. However, since the microglial cell may itself be impaired by such •NO concentrations, it appears first to render itself resistant to the effects of •NO: these measures are instituted in response to even low LPS concentrations (222). Furthermore, and presumably in an effort to avoid the toxicity of peroxynitrite, it appears that cells which can produce both superoxide and •NO tend to stagger their production so that both radicals are not produced at the same time. In part this may be achieved by the inhibition by •NO of the factors involved in superoxide production, and in part by the fact that the agents which stimulate •NO production typically do not also stimulate superoxide production (see (44) for discussion). However, it seems likely that this ingenious protection will be compromised in areas of inflammation, populated as they are by different types of cell presumably sometimes at different stages of activation. If so, then peroxynitrite formation may be anticipated in inflammatory lesions within the nervous system, and, indeed, there is evidence for this in MS lesions (see nitrotyrosine labelling under "MS" below).

Evidence For The Involvement Of Nitric Oxide In Demyelination. The diversity of interactions between

the host of inflammatory mediators make it difficult to establish a direct and causal link between •NO and demyelination. For example, even if the inhibition of •NO production can be shown to prevent demyelination, •NO may not act directly to damage myelin or myelinating cells, but rather act by the induction or enhancement of other factors. However there is substantial indirect evidence that •NO at least participates in demyelination, from work on a number of demyelinating diseases and disease models.

Experimental Autoimmune Encephalomyelitis And Neuritis. Experimental autoimmune encephalomyelitis (EAE) and neuritis (EAN) are primarily T cell-mediated inflammatory disorders of the CNS and PNS which serve as models of MS and Guillain-Barré syndrome (GBS) respectively. Increased proinflammatory cytokine expression has been demonstrated in both EAE (146) and EAN (220), implying that increased •NO production is likely to occur. Indeed, increased iNOS expression has been demonstrated in the CNS of animals with EAE. By measuring •NO levels directly using electron paramagnetic resonance (EPR), Lin *et al*. (136) found a statistically significant increases in the signal associated with iron-nitrosyl complexes in the spinal cords of mice with adoptive transfer EAE. Exogenously applied spin-traps have also been utilised with EPR to demonstrate increased •NO in the spinal cords of animals with both adoptive transfer (106) and actively induced (105) EAE. Unfortunately, absolute levels of •NO are difficult to determine, but these studies suggest that the level of •NO in the spinal cord in passsive EAE lies between 6 and 30 μM (105, 106). This concentration is high (•NO functions as a physiological messenger at nanomolar concentrations), and higher than that at which •NO has effects on axonal conduction (183) (see below). Using reverse transcriptase polymerase chain reaction techniques (RT-PCR) (55, 130, 167) and ribonuclease protection assay (55), a significant increase (up to 10-30 fold) in iNOS mRNA has been demonstrated in the CNS of animals during the acute phase of adoptive transfer (55) or actively induced (130, 167) EAE. In contrast to animals with passive transfer EAE (55), animals with MBP-induced EAE demonstrate a second phase of iNOS up-regulation during the chronic phase of the disease (167), but this is not matched by a concomitant decline in neurological signs. Immunohistochemical techniques have revealed both increased iNOS expression in the spinal cords of mice with EAE (57, 167, 235), and immunoreactivity for nitrotyrosine, indicating the presence of peroxynitrite and its derivatives in the lesions (56, 57, 235).

Multiple Sclerosis. Indirect evidence that •NO plays a role in MS lesions includes the observations that proinflammatory cytokines including TNFα (104, 201) and IFNγ (36, 233) have been identified in astrocytes within MS lesions. IFNγ (137), IL-1 (149) and TNFα (162) have also been found within peripheral mononuclear cells and cells within the cerebrospinal fluid (CSF), and cultured peripheral monocytes have been shown to express more •NO in patients with active disease (197). Some studies have also shown that nitrite and nitrate (metabolites of •NO) are significantly elevated within the CSF of MS patients (56, 80, 119), and that the levels are directly related to disease state (56, 254). However, other studies found no such evidence in either MS or GBS (114).

There is, however, direct evidence that •NO is produced within MS lesions. iNOS mRNA is elevated in MS plaques (17), and is located chiefly in cells of the monocyte/microglial lineage (8, 62, 105). Dual-label histochemistry using anti-NOS antibodies and cell specific markers has shown that macrophages/microglia within actively demyelinating lesions express high levels of both iNOS (8, 62, 105) and cNOS (62). Astrocytes in active MS lesions have been reported to express cNOS (62) rather than iNOS (62, 105), and their expression of NOS is consistent with their positive staining for NADPH-diaphorase (17, 31, 62). Finally, as noted above, •NO can combine with superoxide to form peroxynitrite, and although this may be relatively short lived within lesions, it (or its derivatives) can nitrate tyrosine to produce the relatively stable "footprint" molecule nitrotyrosine. Increased nitrotyrosine reactivity is present in MS brains (8, 105), particularly in areas of demyelination and inflammation (56). Taken together these studies demonstrate the presence of increased production of •NO within MS lesions.

Viral Demyelinating Disorders. There is also direct evidence of •NO involvement in a viral disorder which has a demyelinating component. In mice infected with the coronavirus mouse hepatitis virus strain JHM (MHV-JHM), there is an acute, non-demyelinating phase, and a chronic, demyelinating phase. During the demyelinating phase, *in situ* hybridization and immunohistochemistry have demonstrated that iNOS expression is up-regulated in astrocytes in and around the demyelinated lesions (89, 224).

Consequences Of Nitric Oxide Production On Cells Within The Nervous System. Although the evidence presented above indicates that •NO production is increased in inflammatory demyelinating diseases, the

relative contributions made by •NO, compared with the derivatives of •NO, to pathophysiology remains unclear. Increasingly, it is felt that many of the actions formerly ascribed to •NO may, in fact, be mediated by short-lived, but highly reactive, molecules, such as peroxynitrite and its derivatives. Significant amounts of peroxynitrite are likely to be generated, since every 10-fold increase in •NO and superoxide formation leads to an approximately 100-fold increase in peroxynitrite formation (180). There is also evidence *in vitro* that where the concentration of the arginine substrate of NOS is low, the NOS enzyme can produce superoxide, resulting in peroxynitrite-mediated cytotoxicity (252). Certainly, •NO toxicity is often enhanced considerably by the accompanying presence of superoxide (21, 169), and hence the presumed formation of peroxynitrite.

•NO or peroxynitrite can have a wide variety of effects on cellular systems by modifying protein structure, and thereby function. The two main target sites for •NO in the cell are thiols and metalloproteins. Nitrosylation of thiols to yield nitrosothiols (R-SH + •NO = R-SNO + H^+ + e-) occurs under physiological conditions (217) and appears to by controlled by the relative rates of production of •NO and superoxide (246). Nitrosothiols can sometimes act like •NO in biological systems, and they can function as temporary stores of •NO, effectively increasing its half-life. Although thiol interactions occur more readily, peroxynitrite (or a derivative from it) is also capable of nitrating tyrosine to form nitrotyrosine, and it has been reported that this reaction can be catalyzed by metalloenzymes such as SOD (115). Peroxynitrite can also nitrate tryptophan residues (3), although the physiological significance of this reaction is not known. Beckman *et al*. (11) have pointed out that even apparently simple reactions, such as the nitosylation of thiols by •NO, may in fact be quite complex and involve the production of intermediates such as the nitrosonium ion.

•NO has been shown to inhibit several enzymes, including protein kinase C (81), and enzymes involved in mitochondrial respiration including aconitase, NADH-ubiquinone oxidoreductase and succinate-ubiquinone oxidoreductase (216). Production of endogenous •NO has been shown to inhibit mitochondrial respiration in cultured rat astrocytes (32). This is reflected in an inhibition of the mitochondrial respiratory chain enzyme nicotinamide dinucleotide-dehydrogenase in CNS macrophages/microglia isolated from animals with EAE (263). These actions on multiple members of the mitochondrial respiratory chain may be expected to cause deficits in cellular energy supplies, and indeed ATP content is reduced in neurons exposed to •NO. •NO also affects the activities of several of the enzymes involved in oxidative defence, including catalase, glutathione peroxidase, and Mn-SOD which are all inhibited in C_6 cells by exposure to an •NO donor (68).

In addition to these effects, reactions of proteins with •NO/peroxynitrite may have adverse consequences via the production of neo-epitopes which may provoke an immune reaction, including the production of antibodies. There is evidence to suggest that this phenomenon can occur in MS since some patients show a significant IgM antibody reaction to S-nitroso-cysteine (27).

Peroxynitrite can lead to cell death by several mechanisms, including the nitration of tyrosine residues thereby affecting signalling, direct interaction with DNA (for review see (225)), or by causing DNA strand breakages or deamination. Such breakages result in activation of the enzyme poly-(ADP)-ribose synthetase, which in turn leads to rapid depletion of cellular stores of NAD^+ and ATP (34), and potentially to cell death. •NO can also damage DNA directly by deamination (248), and inhibit the repair activity of the enzyme DNA ligase (85).

Both •NO and peroxynitrite can affect lipid peroxidation (179), and this is an important consequence of •NO production since lipid peroxidation can affect membrane fluidity and membrane permeability, and it can alter the function of proteins embedded in the lipid bilayer. •NO and peroxynitrite can have opposing effects on lipid peroxidation, depending upon the circumstances. If superoxide production exceeds that of •NO, peroxynitrite is formed and this promotes lipid peroxidation (179, 190). If, however, the concentration of •NO is high, lipid peroxidation is decreased because •NO serves as a potent terminator of the radical chain propagation reactions involved. •NO has this effect because it reacts directly with the alkoxyl and peroxyl radical intermediates formed during lipid peroxidation (179). •NO was not found to induce lipid peroxidation, although this role has been claimed in some literature. The data show that •NO formation can therefore have either prooxidant or antioxidant consequences depending upon the concentrations of superoxide and •NO. This diversity of action may help to explain the different effects observed in therapeutic trials based on •NO inhibition in experimental models of inflammatory demyelination (see “Antioxidant Therapy” below).

Gangliosides often show neuroprotective properties, and it has been suggested that this effect may be due to the inhibition of •NO formation (61). In support of this theory, the neuroprotective effects of gangliosides paral-

lel their potency in binding calmodulin *in vitro* (61), and thereby blocking •NO formation.

Sensitivity of Oligodendrocyes and Axons to •NO. Experiments with cultured oligodendrocytes by Merrill and colleagues (145) have yielded the potentially very important observation that these cells are more susceptible to •NO-mediated damage than are astrocytes or microglia. Furthermore, activated microglial cells appear to be able to produce sufficient •NO to lyse oligodendrocytes in co-culture, and this lysis can be prevented by antagonists of •NO production. It may be significant that oligodendrocytes are more sensitive to •NO-induced single stranded DNA breaks than are astrocytes or microglia (153), although oligodendrocyte death apparently occurs by necrotic, rather than apoptotic mechanisms (154).

Although MS is primarily a demyelinating disease, an important cause of the permanent disability in progressive disease is believed to be axonal loss. It may therefore be of interest that axonal loss appears to be related to the degree of inflammation in lesions (232), and that our recent studies have shown that •NO may play a role in axonal loss (213). This role may be accentuated if •NO exposure occurs in conjunction with physiological levels of axonal impulse activity (213).

Effects of nitric oxide on ion channels and the electrophysiological function of axons. In recent years it has become clear that the inflammatory response can play an important part in the pathophysiology of inflammatory demyelinating diseases (156, 258). The data suggest that inflammation can cause axonal conduction block, and that this can lead to significant symptomatology in these conditions. How inflammation causes conduction block in human disease is not known, but recent studies in our laboratory (183) have demonstrated that •NO, or its derivatives, can block conduction in central and peripheral axons *in vivo*, and similar observations have been made by Shrager's group *in vitro* (204). Interestingly, we found that demyelinated axons were especially vulnerable to •NO -mediated block, and that this effect was observed at concentrations of •NO anticipated at sites of inflammation (183). These observations raise the intriguing possibility that •NO production may be responsible for some of the symptoms in MS and other inflammatory demyelinating disorders. If so, then strategies to lower •NO concentrations may form an effective therapy for patients with MS. The mechanism(s) underlying •NO -mediated conduction block are not yet known, but may involve direct effects of •NO on ion channels. The redox state of thiols is well known to affect the conduction properties of axons (reviewed in (188)), and several studies have shown that •NO can act as a modulator of ion channel currents. Consistent with such findings, •NO has been found to inhibit both action potential discharge (143) and sodium currents in baroreceptor neurons (135). •NO also affects currents through Ca^{++}-dependent potassium channels (22), photoreceptor Ca^{++} channels and glutamate ionotropic channels (71). Actions of •NO involving cGMP are well known (74), but these effects appear to be independent of cGMP, and are apparently due to direct interaction with the channel (see also (39)). Surprisingly, the block of axonal conduction observed *in vitro* was dependent upon an intact nerve sheath (204). This observation argues against a direct effect of •NO on ion channels, and the authors proposed a mechanism where an endoneurial intermediate molecule may react with •NO and then with the ion channel.

Taken together, the findings from this and the preceding section suggest that •NO may play an important role in MS, possibly affecting demyelination, conduction block and axonal loss.

The Role Of Reactive Oxygen Species In Demyelination

Production Of Reactive Oxygen Species. In common with other tissues, neural tissues generate ROS constantly as part of their normal functioning. Potential sources of ROS include enzymatic pathways in the mitochondrial electron transport chain, xanthine oxidase, NADPH oxidase, lipoxygenase and cyclooxygenase, as well as non-enzymatic mechanisms such as the auto-oxidation of dopamine and noradrenaline. Mitochondrial respiration is an important source of ROS within the brain, since this organ utilises approximately 20% of the total oxygen taken in each day, and approximately 3% of this is converted to superoxide. Peroxisomes are another source of ROS, and they are abundant in oligodendroglial cells during the period of active myelination.

Although resting microglia produce only small quantities of ROS, this can increase substantially upon their activation (e.g. with IFNγ (251) or phorbol myristate acetate (196), and it may be significant that microglia can become highly activated in inflammatory demyelinating lesions. Cultured human microglia activated by phorbol myristate acetate are especially potent in superoxide production in comparison with other species (43) see also (227), and production is significantly further increased if the microglia are "primed" by exposure to the pro-inflammatory cytokines IFNγ or IL-1α (46):

such exposure would be expected in an inflammatory lesion. The superoxide is produced largely extracellularly as part of the "respiratory burst" phenomenon common to macrophages, neutrophils etc, and mediated by the membrane-bound NADPH oxidase (98, 196), although other generators may also be involved (44). Cells capable of significant superoxide production via the respiratory burst can be recruited to lesions within the nervous system in inflammatory demyelinating diseases. Phagocytic cells are known to be closely associated with myelin sheaths in some such disorders and may act as a concentrated source of ROS. The ROS and RNS formed may directly damage the myelin sheath, promoting attack by macrophages. Indeed, peroxynitrite converts low density lipoprotein to a form recognised by the macrophage scavenger receptor (84).

Neurons may make an important contribution to ROS production, since it is known that neuronal electrical activity can promote the formation of ROS (23). Important mechanisms probably include the direct transaxolemmal entry of calcium ions as part of excitatory activity, and the release of calcium from intracellular pools. The calcium ions can activate phospholipase A2, leading to the release of arachidonic acid and the initiation of the cascade resulting in the formation of prostaglandins, leukotrienes and thromboxanes by cyclooxygenases and lipooxygenases. These enzymes utilise molecular oxygen, and can generate ROS as they function. It may be significant in this respect that axonal activity may be greater than normal in demyelinating diseases such as MS. The increase can result either from the development of sustained, spontaneous, repetitive discharges in demyelinated axons (122, 214, 212), or from the enhanced firing rates of spared axons presumed to occur in compensation for the function lost in axons blocked by demyelination (211). Massed synchronous discharges have also been reported. Electrical activity, especially the massed activity which has occasionally been reported in demyelinating disease (212), is also accompanied by a significant increase in extracellular potassium concentration. Elevated potassium levels may be predicted to be especially large in those MS lesions which have a reduced density of astrocytes (e.g. "open" lesions (9)), since these cells are believed to buffer the extracellular potassium concentration. The raised extracellular potassium concentration may raise ROS levels further since it has been found to increase superoxide production by cultured microglia activated by exposure to phorbol myristate acetate (45).

Inflammatory demyelinating lesions, such as those in MS and GBS, are also likely to contain T lymphocytes among the inflammatory infiltrate. These cells have not been shown to produce ROS as part of their normal function, but a modification of their mitochondria can result in superoxide production during the early stages of apoptosis (142, 260). Since these cells are not known to produce $^{\bullet}NO$ it is interesting that $CD4^{+}$ cells can show immunoreactivity for nitrotyrosine in EAE lesions (57). Nitrotryrosine is a marker for the former presence of peroxynitrite, and it is possible that this potent oxidising agent is formed by the combination of $^{\bullet}NO$ diffusing from nearby cells reacting with superoxide produced by imminently apoptotic T cells (57).

Mature oligodendrocytes are not believed to be major producers of ROS, but increased ROS formation is likely to occur in oligodendrocytes during myelination. This increase arises both because of the energy demands of myelin formation, and because myelin synthesis involves lipid synthesis in peroxisomes. These organelles increase in number during myelinogenesis (5), and they can produce significant quantities of superoxide, and thereby the production of hydrogen peroxide through the action of SOD ((234) see also below).

Evidence For Increased ROS Production In Inflammatory Demyelinating Disease. There is convincing evidence that ROS production is a prominent feature of inflammatory demyelinating diseases. The evidence is derived from observations in animal and human disease, and by inference from the success of several therapeutic trials based on the manipulation of ROS production (see "Antioxidant Therapy").

Lipid Peroxidation. Evidence of ROS generation in the brains of patients has been derived either indirectly, from measurements of the products of lipid oxygenation during the course of the illness, or directly, after death. The indirect evidence has been partly conflicting. An early study found evidence of lipid peroxidation in the CSF of patients with MS, but not in the plasma (111), but a subsequent study found evidence in the serum, but not the CSF (158) see also (81). In the latter study (158), no correlation was identified between levels of lipid peroxides and disease severity or time since relapse. However, as the authors pointed out, few of their patients had severe disability or a long relapse-free interval. The authors considered that elevated CSF levels of peroxidation products were absent either because there was no increase in CSF lipid peroxidation or, more likely, because CSF lipid peroxides were removed rapidly following their generation. Another study (35) found evidence of significantly higher concentrations of malondialdehyde in patient CSF, together with altered levels

of enzymes involved in oxidative defence: the activity of glutathione reductase was significantly increased, whereas the activity of glutathione peroxidase was markedly decreased. In a recent study, lipid peroxidation in plasma and CSF samples were not detected above control levels in patients with MS or aseptic meningitis, but a significant increase was detected in plasma samples from patients with GBS (91). The authors drew a tentative correlation between this finding and the observation that plasma exchange can shorten the course of disease in GBS. Raised concentrations of the antioxidant protein haptoglobin were detected specifically in GBS, but no significant increase was detected in lipid peroxidation in the CSF of GBS patients (91). Evidence of lipid peroxidation was sought in another study by measuring the breath levels of pentane (derived from linoleic acid) and ethane (derived from linolenic acid) as markers (231). Ethane levels were found to remain stable, but pentane rose significantly during acute exacerbations of MS. Excretion of pentane subsequently fell when patients entered clinical remission. However, it has subsequently been claimed that all measurements of breath pentane may be invalid due to technical artifacts (215).

Direct evidence of lipid peroxidation has been demonstrated in post-mortem MS brain. High pressure liquid chromatography was used to demonstrate an increase in uric acid in MS plaques, with a corresponding decrease in glutathione (132). The levels of the lipid radical scavenger vitamin E (α-tocopherol) were lowest in plaques and highest in distant white matter. Newcombe *et al*. (164) used immunocytochemical techniques to detect low density lipoprotein (LDL) and LDL modified by the lipid peroxidation products, malondialdehyde and 4-hydroxynonenal in early and actively demyelinating plaques. Both LDL, which may enter the CNS after blood brain barrier damage in the acute inflammatory lesion, and its oxidation product were found in foamy macrophages within lesions, and also in astrocytes. These findings point to lipid peroxidation as an early event in the evolution of the plaque. Further direct evidence of oxidative damage to lipids, and proteins, in MS lesions has recently been detected using Fourier transform infrared microspectroscopy (134). Data were obtained with near microscopic resolution, and revealed that while normal areas of MS white matter had similar spectra to control white matter, spectra from within MS lesions indicated an increase in the C=O to CH_2 ratio, suggesting lipid oxidation. Other data were consistent with the oxidation of proteins (134). The brain tissue had been fixed in formalin, but control studies suggested that fixation itself did not affect the data obtained. The failure of an earlier, similar study (40) to detect changes consistent with oxidative damage was attributed to earlier technical limitations, and study of chronic inactive plaques rather than the active plaques studied by LeVine and Wetzel.

Lipid peroxidation can result in the release of a number of membrane components, including arachidonic acid. Arachidonic acid can be converted to prostaglandins by the enzyme cyclooxygenase and also to isoprostanes by the non-enzymatic free radical-catalysed peroxidation of arachidonic acid (241). The isoprostanes, particularly 8-epi-$PGF_{2\alpha}$, are used as indicators of oxidative stress (64, 185). The production of these pro-inflammatory compounds may evoke further tissue damage. For example, it appears that prostaglandin E_2 can act in conjunction with $^{\bullet}NO$ to disrupt the blood brain barrier (117). A role for oxidant species in barrier breakdown is supported by the observation that administration of a 21-aminosteroid antioxidant (one of a group of compounds known as "lazaroids") can attenuate barrier disruption induced by arachidonic acid (96). The prostaglandins are not necessarily damaging to tissues, however. Administration of a long-acting analogue of prostaglandin E_1 has been found to suppress EAE (182). The details concerning the effects of ROS on the blood-brain barrier are the subject of recent reviews (63, 147).

Other evidence for ROS production. Other evidence for ROS production in MS has centred on the examination of blood cells obtained from patients. Studies in 1986 found that the antioxidant enzymes SOD (4) and glutathione peroxidase (118) were lower in erythrocytes and haematogenous cells, respectively, of patients than neurological controls. In keeping with an altered redox state, it has been found that red cell glutathione peroxidase levels are depressed in MS, with similar reductions occurring in circulating lymphocytes and granulocytes (205). Furthermore, stimulated monocytes from patients with MS were found to produce significantly more hydrogen peroxide and superoxide than monocytes from controls (73). The authors concluded that blood monocytes in MS patients are "primed" so that they produce more ROS than normal when exposed to inflammatory stimuli. A subsequent study (81) obtained similar results using whole blood. A finding that red blood cells in patients with MS displayed increased mechanical fragility has been attributed to free radical-mediated damage to lipids in the red cell membranes (37, 198).

Several studies have examined ROS production in experimental models of inflammatory demyelinating disease. A role for hydrogen peroxide was suggested by findings in actively-induced EAE (93). The evidence

suggested raised hydrogen peroxide concentrations in the myelinated portion of the optic nerve in the early preclinical phase of the disorder when the blood brain barrier was expected to be initially disturbed. The authors proposed that the hydrogen peroxide acted both alone, and through the generation of free radicals in a cascade of lipid peroxidation and demyelination. Examining rats with clinical signs of EAE, Ruuls and colleagues found that macrophages and microglial cells exhibited significantly higher spontaneous levels of ROS compared with controls (192). Similarly, and examining the same model, a later study found significantly higher levels of superoxide in all the CNS regions examined (200). Where superoxide is formed in the presence of •NO, the agents combine to form the strong oxidising and nitrating agent peroxynitrite, and a recent study found evidence that peroxynitrite is formed very early during the course of EAE, correlating with disease activity (235). The labelling was present in macrophages/microglia, which also showed iNOS reactivity.

Consequences Of ROS Production. An important consequence of ROS formation is the promotion of inflammation via effects on endothelial cells, chemotactic signals and the release of products from the arachidonic acid cascade. These effects are beyond the range of this review, but inflammation can affect the progression of demyelinating lesions, and perhaps its immunological consequences.

Effects Of ROS on Oligodendrocytes. The death of oligodendrocytes, the central myelinating cell, is an early event in many demyelinating lesions in MS (33, 176), but the circumstances which cause the death of the oligodendrocyte remain uncertain. This uncertainty applies even though MS is believed to be an autoimmune disease directed against myelin or the myelinating cell. There is now evidence that ROS may play a role in oligodendrocyte death, in addition to the potential role for •NO described above.

Several studies have examined the sensitivity of cultured oligodendrocytes, and their precursors, to damage by ROS. These studies followed observations by Griot and colleagues that the demyelination caused in dogs by infection with the canine distemper virus may be due to ROS released by brain macrophages (86, 87). The culture of dog oligodendrocytes in the presence of ROS generated by the xanthine/xanthine oxidase reaction (an experimental system to generate superoxide), revealed that the oligodendrocytes were killed at ROS concentrations which did not appear to affect astrocytes or brain macrophages (88). Similar findings were subsequently made with bovine oligodendrocytes, which could be completely protected from death by the inclusion in the medium of catalase, an enzyme which degrades hydrogen peroxide to water, but not by the inclusion of the antioxidants SOD, DMSO, vitamin E or glutathione (124). The implication that hydrogen peroxide was responsible for the damage was consistent with findings from other cultured cell types and was also implicated in the toxicity of oligodendrocytes, from adult rat brain, arising from exposure to the catecholamines norepinephrine and epinephrine (165). Catecholamine metabolism can generate ROS, including hydrogen peroxide, and it is significant that the catecholamine toxicity could be prevented by catalase, and reproduced by the addition of equimolar concentrations of exogenous hydrogen peroxide. However, the toxicity is not necessarily due to hydrogen peroxide directly, even though it is a mild oxidant, since the toxicity may rather be due to the formation of the highly toxic hydroxyl radical through the action on hydrogen peroxide of transition metals such as iron and copper (98): oligodendrocytes are particularly rich in iron (see below).

Hydrogen peroxide is also produced in quantity in peroxisomes (234). Peroxisomes are particularly abundant in oligodendrocytes during the period of active myelination (5), raising the possibility that oligodendrocytes may be particularly vulnerable to oxidative stress during this period. If so, then oligodendrocytes may also be vulnerable during the repair, by remyelination, of demyelinating lesions in MS and other demyelinating disorders (176, 177). In contrast to normal development, repair by remyelination in MS occurs in the context of an on-going inflammatory disease involving increased levels of ROS production. Indeed, repair by remyelination can be observed in conjunction with on-going inflammation (181). It is therefore possible that in response to the combined demands arising from remyelination and exogenous ROS production, some oligodendrocytes may undergo degeneration, contributing to the long term failure of repair by remyelination in this disease.

Apart from having a direct effect on oligodendrocytes, ROS can also directly affect both the lipid and protein components of myelin (66, 129). Thus the incubation of myelin with ROS generated *in vitro* resulted in lipid peroxidation and the decompaction of myelin lamellae along the intraperiod line (24). The incubation also caused the marked peroxidation of myelin basic protein and proteolipid protein, and this rendered the proteins susceptible to trypsin degradation (25). It is

therefore possible that in inflammatory demyelinating disease, exposure to ROS may render myelin susceptible to degradation by extracellular proteases, such as those liberated by macrophages. The decompaction of the lamellae may also facilitate access of proteases to the myelin proteins (25).

An unexpected consequence of ROS/RNS production is the release of active matrix metalloproteinases (MMPs) from their proenzyme form, via the formation of either peroxynitrite or the nitrogen dioxide radical (140). Moreover, hydrogen peroxide exposure has been found to cause an increase in the mRNA and protein expression for MMP-1 (interstitial collagenase) in cultured human fibroblasts (29). The formation and release of MMPs may be relevant to demyelination since some MMPs have been shown to be able to degrade myelin basic protein (38, 77). Furthermore, inhibitors of MMP release have been shown to protect animals from both EAE (52, 76, 103) and EAN (184), the animal models of MS and GBS respectively. However, the net effect of RNS formation on MMP activity is difficult to predict since peroxynitrite has also been found to inhibit some MMPs (169).

Demyelination can also follow ischaemic episodes, or periods of hypoperfusion of the brain. Lesions resulting from hypoxic-ischaemic insult typically involve the grey matter, but delayed white matter lesions are quite common, and primary demyelination (i.e. myelin loss with sparing of axons) can result when the insult affects white matter (78, 79). The consequences of ischaemia are the subject of another review in this symposium, but it is appropriate to mention here the occurrence of primary demyelinating lesions induced in Mongolian gerbils by hyperoxia following transient brain ischaemia (150). This protocol is anticipated to encourage ROS formation, and it resulted in lesions in the corpus striatum, lateral thalamus, mesencephalon and internal capsule. Lesion formation correlated with the administration of 100% oxygen following the transient ischaemia. Delayed primary demyelinating lesions have also been produced in rats and gerbils with chronic hypoperfusion, indicating that mild hypoxia, combined with mild hypoglycemia, can preferentially affect oligodendrocytes (101, 239), reviewed in (120). Interestingly, carbon monoxide exposure results *in vivo* in an increased production of hydroxyl radicals (174), and lipid peroxidation (229), and some patients have been reported to show a delayed primary demyelination following carbon monoxide poisoning (78, 79).

A consequence of ischaemia in grey matter is the release of the neurotransmitter glutamate. Adult rat oligodendrocytes are much more vulnerable to glutamate-induced cell death than astrocytes (succumbing to 200μM glutamate, whereas astrocytes are resistant to 5mM (166)). However, the extent to which oligodendrocytes are exposed to glutamate in demyelinating disease is uncertain. Examination of CSF from patients with MS has resulted in reports that glutamate concentrations can decrease (1, 2, 178), remain unchanged (127), or increase (221). The increased (221) concentration occurred in acute MS patients, and consisted of a doubling of glutamate concentration. Serum/plasma glutamate has more consistently been reported to increase in MS (178), especially during relapses (243). In inflammatory MS lesions, glutamate concentrations might rise since astrocytic glutamate uptake is inhibited by the pro-inflammatory cytokines TNFα, IFNγ and IL-1β, at least *in vitro* (255). Glutamate is toxic to cultured oligodendrocytes through free radical attack consequent to the depletion of cystine, and thereby depletion of the antioxidant glutathione (166). Glutathione depletion consequent to cystine deprivation also kills cultured oligodendrocytes (256). In each case, the cultured oligodendrocytes could be protected by the addition of free radical scavengers, including vitamins C and E.

Particular Sensitivity of Oligodendrocytes to ROS. A characteristic of established lesions in MS is the presence of demyelinated axons embedded among astroglial processes, with few, if any, oligodendrocytes. A factor contributing to this appearance may be the much greater resistance to ROS of astrocytes, in comparison with oligodendrocytes. It appears that several factors contribute to the comparative sensitivity of oligodendrocytes to ROS. For example, recent studies have revealed that cultured oligodendrocytes at several stages of differentiation (proliferative oligodendrocyte progenitor, proliferative oligodendroblast and mature oligodendrocyte) have less than half the glutathione content of astrocytes (121, 230). A low level of this important antioxidant is also apparent *in vivo* (210), and appears to be due in part to a low rate of glutathione synthesis, and in part to their having only half the glutathione reductase activity of astrocytes, and a particularly low level (15% of astrocytic) of glutathione peroxidase activity (121). In addition, cultured oligodendrocytes fail to express Mn-SOD, even when exposed to ROS, in contrast to microglia and some cultured astrocytes (175). Furthermore, immunocytochemical studies have revealed the absence of metallothionein in oligodendrocytes (159, 6), although appreciable quantities are present in astrocytes (15, 16, 160). Metallothionein is a protein particularly rich in cysteine (25-30%), making it an effec-

tive antioxidant and binding agent for zinc and copper (70). Metallothionein is induced by cytokines, particularly IL-1 (123), and it is expressed at high concentration in reactive astrocytes *in vivo* (60, 163). The absence of Mn-SOD and metallothionein in oligodendrocytes removes two more avenues of antioxidant defence from this cell.

A Role For Iron. The increased oxidative risk experienced by oligodendrocytes by virtue of their relative paucity of antioxidant defence is exacerbated by the fact that these cells contain most of the iron in the brain (48-50, 75). It is well known that reduced iron, probably in low molecular weight complexes such as iron-ADP and iron-citrate, can promote oxidative damage *in vitro* by catalysing the formation of hydroxyl radicals from hydrogen peroxide and causing secondary initiation of lipid peroxidation. Most of the iron in cells is in a "safe" form attached to the binding proteins transferrin and ferritin (48-50, 75), and in this form it is not available to catalyse free radical reactions. However, if this iron were to be released it could catalyse peroxidative chain reactions that could spread the effects of the injury over a wider area. It is notable in this regard that superoxide (and other reducing compounds such as vitamin C) can release iron ions from ferritin, and that hydrogen peroxide can release iron from haemoglobin (at high hydrogen peroxide:haemoglobin ratios (92)). Thus superoxide and hydrogen peroxide can create the conditions that lead to hydroxyl radical production, although the extent to which this occurs *in vivo* is not clear. *In vitro*, it has been demonstrated that activated microglia can release iron from ferritin, and that the release of iron is mediated through superoxide production by the microglia since it is blocked by SOD (257). Interestingly, the oxidative stress experienced by cultured oligodendrocyte precursors when exposed to blue light (which excites compounds such as riboflavin ultimately resulting in increased hydrogen peroxide production) could be prevented either by chelating intracellular free iron, or by raising the concentration of intracellular glutathione to astrocytic levels (230).

Oligodendrocyte precursor cells appear to be particularly sensitive to ROS in comparison with astrocytes, and in mixed glial cultures they are preferentially damaged by measures designed to increase intracellular ROS production (112). Preoligodendrocytes also appear to be significantly more sensitive than mature oligodendrocytes to oxidative stress (7).

Although there is agreement that oligodendrocytes are more vulnerable to oxidative stress than astrocytes (245), a recent study found that cultured mouse astrocytes were “exquisitely sensitive” to oxidative stress, and that their vulnerability was related to, and enhanced by, iron (187).

Much of the work cited above was performed *in vitro*, with purified cell cultures. Caution is always required before extrapolating *in vitro* data to the condition *in vivo*, but it is particularly so here since there is evidence that oligodendrocytes may not be as sensitive to ROS *in vivo* as the *in vitro* data suggest. Thus it has been found that the inclusion of a monolayer of neonatal rat astrocytes completely prevented the catecholamine toxicity described above (165): astrocyte conditioned medium provided no protection. Also, neurons co-cultured with astrocytes have markedly elevated concentrations of the antioxidant glutathione when compared with neurons cultured alone (20, 194). Oligodendrocytes *in vivo* may therefore be more resistant to oxidative stress than the available data suggest.

Using histochemical techniques which can detect non-heam iron, deposits of iron have been found in post-mortem MS plaques in some cases. For example, using methods to increase the sensitivity and accessibility of tissue iron to histochemical reagents, iron deposits have been found to occur frequently in post-mortem MS brain. The iron is seen in controls and pathological tissues in both oligodendrocytes and in myelin, but in plaques it is also present in reactive and amoeboid microglia and in macrophages (133). In one out of five cases, iron was also detected in some axons near damaged areas of the white matter. It appears, therefore, that iron is present at sites where it could promote oxidative damage in the brains of patients with MS.

An early report suggested that actively-induced EAE could be suppressed by treatment with the iron-chelating agent desferrioxamine B mesylate (28) see also (94). A later study found that passively-induced disease could not be suppressed in this way (244). A potential explanation for these different findings was that the chelating agent was actually working by suppressing antigen presentation in the afferent limb of the autoimmune response (244). However, a recent finding has found a reduction in clinical signs when animals were treated with desferrioxamine during the active stage of MBP-induced disease (172).

Interaction Between Oxidative and Nitrative Stress. Although the combination of $^{\bullet}$NO and superoxide to form peroxynitrite is well established, it has also been reported that $^{\bullet}$NO can actually reduce superoxide production arising from the respiratory burst (41, 126). This reduction is not due to scavenging of the superoxide, but rather to some other mechanism, which appears not to be mediated by guanylyl cyclase or cGMP. It has been noted that the reduction of superoxide generation may serve to limit the deleterious effects of excessive peroxynitrite forma-

tion (126). This potentially beneficial effect of •NO is counterbalanced by the fact that •NO can decrease the activity of some of the enzymes involved in anti-oxidant defence in cultured oligodendrocyte-like cells. Thus, although exogenously or endogenously produced •NO increased the activity and protein level of Cu/Zn-SOD, it was found to decrease the activities of the antioxidant enzymes glutathione peroxidase, catalase and Mn-SOD, probably through the down regulation of the expression of their mRNAs (68). •NO may also inactivate glutathione peroxidase directly by modifying essential cysteine-like residues in the enzyme. Such observations indicate that •NO production may affect the level of oxidative stress and the effects of ROS production. If •NO reduces the antioxidant defence of oligodendrocytes in early inflammatory MS lesions, namely a location where the cells will be exposed to a range of ROS and RNS, it would provide additional explanation for the loss of these cells and the formation of a demyelinating lesion.

Beneficial Effects of ROS and RNS. This review has focused on the deleterious role of ROS and RNS in promoting demyelinating pathology, but it should be appreciated that these species also serve many beneficial, physiological functions (e.g. •NO functions as a messenger by binding to guanylate cyclase) which are beyond the scope of this review (see (10, 74, 98, 249)). •NO, in particular, may also serve beneficial functions under pathological circumstances. It can, for example, react with organic radicals and it can stop chain radical reactions. Perhaps as a consequence of such reactions, several experiments have found that blocking •NO production in EAE is sometimes detrimental (see "Antioxidant Therapy" below). Indeed, •NO is known to exhibit some anti-inflammatory properties, such as decreasing the proliferation of lymphocytes *in vitro* (65), inhibiting the adherence of leukocytes to the vasculature and infiltration into the tissue (131), and decreasing IFNγ production by Th1 T lymphocytes (228). Other protective effects of •NO include protection from superoxide- or hydrogen peroxide-mediated damage to cells *in vitro*, perhaps achieved by blocking the formation of hydroxyl radicals (247).

Antioxidant Therapy. As reviewed above, there is substantial evidence that RNS and ROS are involved in demyelination, and in demyelinating disease. This evidence has spawned a number of examinations of antioxidant and antinitrative therapies, both *in vitro* and *in vivo*, as described below.

We have already mentioned the observation that oligodendrocytes *in vitro* are very sensitive to the effects of ROS, and that they can be protected from ROS- and catecholamine-induced cell death by the addition of catalase to degrade hydrogen peroxide (124, 165). Oligodendrocytes can also be rescued from glutamate-mediated death by supplementation with cystine or cysteine (166). Also, another antioxidant (N-acetyl-L-cysteine (NAC), which can effectively raise intracellular glutathione levels) is able to protect cultured oligodendrocytes from toxicity mediated by the pro-inflammatory cytokine TNFα (144): there is evidence that TNF toxicity in culture is mediated via ROS (199, 250). Such observations encourage a belief that antioxidant therapies may be of value in demyelinating diseases such as MS, and this possibility has been examined *in vivo* using animal models.

Hartung *et al*. (100) found that early treatment of rats with the antioxidants catalase or SOD protected against actively-induced EAN, and that even if treatment was delayed until after the onset of neurological deficit (i.e. a clinically relevant regimen) the agents were still effective in markedly reducing the severity of disease. This important demonstration that ROS may be involved in peripheral autoimmune demyelinating disease was followed in 1992 by the observation that one of the 21-aminosteroid antioxidants was effective in reducing the incidence and severity of actively-induced EAE in the CNS (95). The administration of catalase before the onset of neurological deficit was also found to delay the onset of EAE and to reduce its severity and duration (192). Furthermore, another antioxidant, butylated hydroxyanisole, was effective in reducing the incidence, severity and mortality of passively-induced EAE (99). A synthetic catalytic scavenger of oxygen radicals, EUK-8, also ameliorated EAE in mice (141). This evidence for the involvement of ROS was accompanied in 1994 by the demonstration that RNS may also be involved. Several investigators have shown that aminoguanidine, a weak but preferential inhibitor of the inducible/inflammatory form of NOS, inhibits the expression of disease in mice and rats with actively-induced EAE (30, 58, 261) and inhibits demyelination induced by Theiler's murine encephalomyelitis virus (189). Also, the use of antisense knockdown of iNOS in mice has been shown to inhibit EAE (67). Additional data supporting a view that decreasing •NO concentrations may be of therapeutic value have been presented by Hooper *et al*. (105, 107). These authors found that the inhibition of iNOS induction (using tricyclodecan-9-xyl-xanthogenate), or scavenging of •NO (using 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) or peroxynitrite (using uric acid), inhibited neurological disease in mice

with actively-induced EAE. Withdrawal of the iNOS inhibitor resulted in the expression of neurological signs within 24 hours.

The evidence described above provides a seemingly unambiguous argument for the value of antioxidant therapy in inflammatory demyelinating disease. However, data reported in 1995/6 (193, 262) suggested that the inhibition of nitric oxide production is not always beneficial, and the most recent data (54) indicates that such inhibition can be lethal. Zielasek and colleagues (262) used several inhibitors of nitric oxide synthase, namely NG-L-monomethyl arginine (L-NMMA), which is relatively non-specific for different NOS isozymes, aminoguanidine, which is relatively selective for the inducible form of NOS, and two other inhibitors. They examined the effects of these inhibitors in different models of experimental autoimmune disease. L-NMMA partially suppressed passively-induced EAN (i.e. EAN induced by the injection of neuritogenic T-cells), but not actively-induced EAN or passively-induced EAE. Aminoguanidine enhanced actively-induced EAE, and had no significant effects on actively-induced EAN. The other NOS inhibitors had little or no effects in EAN and EAE. As the authors noted, the diversity of action indicates that the •NO pathway may play a more complex role in disease than previously suggested. Ruuls *et al*. (193) found that the NOS inhibitors N(omega)-nitro-L-arginine and N(g)-monomethyl-L-arginine, exacerbated actively-induced EAE. Another study (54) also found that NOS inhibition, by aminoguanidine, exacerbated actively-induced EAE. Other recent data has revealed that iNOS knockout mice have more severe EAE (72, 195). Indeed, the complexity was highlighted further by the observation that another NOS inhibitor, N-methy-L-arginine (NMA), could induce disease in PVG rats which are normally resistant to actively-induced EAE (54). The disease in PVG rats was fulminating in nature, and accompanied by some mortality. Interestingly, in the absence of NOS inhibition, PVG rats were found to develop higher serum nitrite and nitrate levels (surrogate markers of •NO production) than did Lewis rats, even though Lewis rats are susceptible to EAE. This data suggested that •NO may actually protect PVG rats from disease, and additional *in vitro* data indicate that this protection may be due to the inhibition by •NO of T cell proliferation (54).

A role for antioxidants in MS has been suggested by many authors e.g. (51, 113, 242), but no large scale trials have been conducted. However, therapy with IFNβ has been shown to reduce the relapse rate and possibly clinical progression (97, 191). It is often proposed that IFNβ produces its beneficial effects by mechanisms involving immune modulation. However, it has recently been realised that IFNβ also impairs the ability of astrocytes *in vitro* to form iNOS in response to inflammatory stimuli, such as exposure to the cytokines IL-1β and IFNγ (90, 109, 218, 219). IFNβ also downregulates the level of steady state expression of iNOS in cultured astrocytes (109). These observations raise the possibility that, in part at least, IFNβ may effectively be functioning in MS as a component of the antioxidant defence. If so, then other therapies based on this strategy may also be beneficial. The role of nitric oxide in MS has been examined in a recent review (171).

The variable observations obtained with the use of NOS inhibitors in experimental autoimmune demyelinating disease may be related to the relative lack of potency and specificity of the inhibitors, or to inopportune timing of the therapy in the course of the disease: •NO has immune regulatory effects as well as cytotoxic ones (see above), and so the timing of administration could be critical. Theory suggests that if •NO inhibition can be an effective therapy for MS, an ideal •NO inhibitor would be one which was specific for iNOS, since this would leave the eNOS and nNOS enzymes unaffected, permitting normal physiological function to continue. [However, it may be worth mentioning evidence that iNOS can be induced in response to non-inflammatory, non-immunologic, or inapparent stimuli, and so even a specific inhibitor may have unwanted "side" effects (see discussion in (161)).] When such an inhibitor is discovered it will be interesting to consider whether it may best be employed in conjunction with neurotrophic therapy. Evidence has recently been advanced suggesting that at least one of the neurotrophins, brain-derived neurotrophic factor (BDNF), limits its own neuroprotective potential by inducing NOS, and thereby damaging •NO production (128) see also (144). The data showed that whereas neither BDNF nor a free radical inhibitor (either the spin trap S-PBN or the NOS inhibitor L-NAME) effected significant survival of retinal ganglion cells from axotomy-induced death, the agents acted synergistically upon co-administration to rescue significant numbers of RGCs.

Hyperbaric oxygen has been advanced as a therapy in MS following positive findings in EAE (240). Clinical trials have failed to demonstrate any beneficial effect (125), but the findings in EAE remain unexplained. It has been reported (4) that exposure of MS patients to hyperbaric oxygen resulted in a significant increase in erythrocyte SOD levels, indicating an increase in cellular oxidative defence. It seems likely that the exposure

to hyperbaric oxygen resulted in an increase in superoxide, and that this induced the formation of oxidative stress enzymes. If such enzymes were also induced in CNS cells, it would increase the tolerance of the cells to oxidative stress and this could help to explain the beneficial effects in animals (242).

Future Therapy of MS and GBS from an antioxidant perspective. It seems likely that peroxynitrite, or one of its derivatives, plays a role in the development of MS lesions, given the presence of nitrotyrosine labelling within lesions (56, 57, 235). This view is supported by the efficacy of a trial therapy based on peroxynitrite scavenging using uric acid (105, 107) (although uric acid actually scavenges several reactive species, not just peroxynitrite). Since peroxynitrite is formed by the combination of superoxide and •NO, peroxynitrite formation can be diminished by inhibiting the production of either of these molecules: it is not necessary to inhibit both. Indeed, strategies aimed at reducing the formation of either molecule have produced beneficial effects in EAE (30, 58, 67, 95, 99, 100, 105, 107, 141, 192, 261). It is too early to state with any certainty whether superoxide or •NO (if either) will form the best target for new therapies in human demyelinating disease. Current electrophysiological evidence from our laboratory shows that exposure to •NO donors can promote conduction block (183) and axonal degeneration (213), and such observations appear to recommend therapeutic strategies based on decreasing the concentration of •NO. However, the effector molecule (whether •NO, peroxynitrite or another •NO derivative) in the electrophysiological experiments is not yet clear. Furthermore, therapies designed to decrease •NO concentrations (54, 72, 193, 195, 262) have been less consistently beneficial in experimental demyelinating disease than those designed to limit superoxide. This latter consideration favours the development of strategies to inhibit extracellular superoxide formation within lesions. Such strategies may also limit the formation of hydrogen peroxide, a molecule which appears to be quite toxic to oligodendrocytes (124, 165). The pace of research into the roles of ROS and RNS in demyelination is accelerating rapidly, and the optimal strategy may soon become clearer.

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