ROLE OF THE IMMUNE RESPONSE IN TISSUE DAMAGE AND REPAIR IN THE INJURED SPINAL CORD

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7.1. Introduction
The immune system has evolved primarily to protect the body from invasion by microorganisms such as bacteria, viruses and parasites, and to contribute to repair processes during disease and after injury. Although the immune response to injury in non-CNS tissue often leads to repair that is desirable, similar responses in the injured mammalian CNS causes gliosis that could be detrimental to the growth of damaged axons and repair of neural circuitry. In addition, these immune responses in the injured CNS have also been implicated in the extensive tissue loss sustained at the site of injury. Despite the evidence that autoimmune responses to self-antigens lead to disease, which most notably in the CNS leads to multiple sclerosis in humans and experimental allergic encephalomyelitis (EAE) in animal models, recent studies suggest that some types of autoimmune responses may be guided to protect the CNS after injury. Other work also indicates that antibody and macrophage responses may also be recruited to promote axon growth and regeneration. These studies appear at a time when other evidence points to the detrimental role of the immune response in neurodegenerative diseases such as Alzheimer’s disease and amyotrophic lateral sclerosis (Giulian 1999, Zhu et al. 2002, Kriz et al. 2002, Wyss-Coray and Mucke 2002). Can the innate and adaptive arms of the immune response be recruited to favor tissue repair and axon regeneration after spinal cord injury?

One of the consequences of spinal cord injury (SCI) is loss of neural tissue at the site of injury and the damage to axons of long fiber tracts that traverse the length of the cord in both directions. Motor fibers descend from cortical and brainstem neurons while fibers carrying sensory information from peripheral receptors, such as those in the skin and from sensory neurons in the spinal cord, ascend to neurons in the brainstem, cerebellum and other regions of the CNS. Damage to these descending and ascending fiber tracts will, therefore, result in loss of motor, sensory, and autonomic functions. Since the CNS environment, unlike that in peripheral nerve is inhibitory for axon regeneration, damage of axons of the long fiber tracts in the CNS leads to permanent functional deficits.

In this review we will focus primarily on SCI. Two aspects of the injury response will be considered: (i) tissue loss and protection at the site of spinal cord lesion, and (ii) axon damage and regeneration. We will assess the evidence for the involvement of the immune response in the generation of the pathological changes at the site of SCI, as well as its potential for influencing tissue protection and axon regeneration.

7.2. Tissue loss and protection
7.2.1. Mechanisms of tissue damage
Two models of SCI are widely used in studies of regeneration and repair: (1) hemisection (dorsal or unilateral hemisections); and (2) contusion injury. The former has been used effectively for many years to study long distance axon regeneration, while the latter has been the preferred model for studies of trauma-induced tissue loss. Experimental spinal cord contusion injuries in rodents show many histopathological and functional similarities to spinal cord injuries most
often sustained in humans. This contusion model has been used to characterize the immediate
damage due to mechanical injury, delayed secondary damage resulting in the progressive death
of the surrounding neural cells by apoptosis, and the formation of cystic cavities (Noble and
Wrathall 1985, Bresnahan et al. 1991, Behrmann et al. 1992, Reier et al. 1992, Constantini and

Results from several studies support the view that the two types of cell death, immediate
and secondary cell death proceed sequentially in the injured spinal cord (Tator and Fehlings
the site of CNS trauma occurring immediately after the injury, whereas apoptosis contributes
more to secondary cell loss at later periods. Apoptotic cells are detected from 4 hrs to at least 3
weeks after spinal cord contusion (Crowe et al. 1997, Liu et al. 1997). Accordingly, the size of
the lesion and the extent of cavitation in rats increase progressively over days to weeks following
SCI (Steward et al. 1999). While it may be unrealistic to hope to prevent much of the rapid
death of neural cells undergoing necrosis due to the direct mechanical effects of the trauma,
potential strategies are currently being tested to prevent secondary apoptotic cell death.
Although the precise mechanisms of secondary damage are still unclear, it is known that the
initial traumatic injury leading to primary cell death is immediately followed by the removal of
tissue debris by inflammatory cells. It has been hypothesized that primary cell death and the
inflammatory response might both play a role in initiating secondary damage via signals that are
still not fully identified.

Among the candidates for mediating secondary toxicity are factors such as calcium,
proteases, glutamate, and other excitatory amino acids, to name a few. Over the last few years,
however, free radicals, cytokines, and chemokines have received greater attention with regard to
their potential contribution as mediators and modulators of secondary CNS degeneration at the
site of trauma. Free radicals, cytokines and chemokines are induced in response to various CNS
insults and can cause cellular injury and apoptotic cell death both in vitro and in vivo (Lewen et
al. 2000, Allan and Rothwell 2001). Notably, levels of free radicals, cytokines and chemokines
are elevated within spinal cord lesion sites following a time-course that fits well with the timing
of secondary cell loss (Hamada et al. 1996b, Bartholdi and Schwab 1997, McTigue et al. 1998,
Inflammatory cells are the principal source of these factors after CNS trauma. What are some of
the roles these inflammatory cells play in CNS trauma, and how can they be manipulated in an
attempt to reduce secondary damage and promote neuroprotection?

The influence and consequences of the inflammatory response following SCI is still a
matter of intense study. Several reports support a deleterious role of inflammation after CNS
1998, Popovich et al. 1999). However, other studies have also raised the possibility that
inflammatory responses after CNS lesions may have some beneficial effects (David et al., 1990,
Rapalino et al., 1998, Hauben et al., 2000, Leon et al., 2000). It is possible that inflammatory
cells could participate in both secondary degeneration and tissue protection, depending on their state of activation at various times after lesion.

7.2.2. Involvement of immune cells and approaches to altering their response
The growing evidence that inflammation contributes importantly to the orchestration and progression or control of secondary tissue damage has paved the way for studies aimed at blocking or stimulating different aspects of the inflammatory response. A number of approaches have been tested thus far to alter the immune cell response in an attempt to obtain tissue protection after SCI. We will discuss these under the headings of cell directed and general approaches to modulate the immune response after CNS injury.

A. Cell directed approaches to modulate the immune response
Blocking immune cell recruitment:
Soon after spinal cord contusion or hemisection, the site of lesion becomes filled with large numbers of hematogenous inflammatory cells. Neutrophils, lymphocytes, and monocytes are successively chemoattracted to this site and occupy the area of the lesion for limited or prolonged periods of time depending upon the cell type (Dusart and Schwab, 1994; Popovich et al. 1997, Carlson et al. 1998, Schnell et al., 1999a, Ma et al. 2002). These cells release and respond to a multitude of soluble signals and organize an inflammatory response that will lead to phagocytosis of tissue debris and pathogens from the site of injury and ultimately to wound healing. Chemokines and cytokines, in particular, are involved in the attraction of blood-derived inflammatory cells into the CNS parenchyma and their activation (Bell et al. 1996, Schnell et al. 1999b, Asensio and Campbell 1999). In general, inflammation is thought to proceed when the balance in the levels between pro- and anti-inflammatory cytokines tilts in favor of the former. Inflammatory molecules released in the CNS parenchyma after an injury may participate in both neuropathological and neuroprotective responses. Interestingly, Bethea and colleagues showed that interleukin (IL)-10, a potent anti-inflammatory cytokine, reduces spinal cord cavitation and improves functional outcome if provided as a single dose after injury, but worsens the outcome if administered on two occasions 48 hours apart after SCI (Bethea et al. 1999).

Since the pathological changes after SCI are constantly evolving in the early period after injury, the role chemokines and cytokines play is likely to depend on the time after injury and the levels of these immune modulators. Along these lines, several studies have reported that macrophage inflammatory protein (MIP)-1α, macrophage chemotactic protein (MCP)-1, and tumor necrosis factor-alpha (TNFα) play a key role in the inflammation and tissue damage that occurs in the CNS in experimental allergic encephalomyelitis (EAE)(Karpus et al. 1995, Karpus and Ransohoff 1998). However, our laboratory has recently shown that MIP-1α, MCP-1, and TNF-α are also key players in lysophosphatidylcholine (LPC)-induced inflammatory responses in the spinal cord that lead to macrophage activation and myelin phagocytosis without causing cell death and tissue damage (Ousman and David 2001). The differences in these two models may lie in the timing and duration of chemokine/cytokine expression. In the LPC model, the
expression of these chemokines and cytokines is limited to a very short period of 1-2 days, while in EAE their expression is prolonged over a period of a week or more (Godiska et al. 1995, Karpus et al. 1995, Glabinski et al. 1997). In addition, in the LPC model, rapid down-regulation of these chemokines and cytokines is accompanied by the expression of anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta (TGFβ) (Ousman and David 2001). The duration of expression of pro-inflammatory chemokines and cytokines and the coordinated expression of anti-inflammatory molecules are key factors in the development of a safe inflammatory response. Similarly, although a number of chemokines and cytokines have been implicated in the pathology after SCI based on expression studies (Schnell et al., 1999b, Bartholdi and Schwab, 1997, McTigue et al., 1998, Ghirniker et al., 2000, 2001), they may exert positive or negative effects depending on the context and time after injury. Inflammatory cells also produce other cytotoxic molecules such as free radicals that could actively participate in secondary degeneration after SCI (Satake et al. 2000, Chatzipanteli et al. 2002). Hydrogen peroxide released after CNS damage can react with ferrous iron to produce highly toxic hydroxyl radicals (Hyslop et al. 1995). The excessive infiltration of immune cells, which contribute to increased levels of pro-inflammatory and cytotoxic molecules, can therefore also lead to cellular damage and tissue destruction after SCI.

**Neutrophils:**
All the evidence points to neutrophils as being one of the first blood-derived inflammatory cells to infiltrate the site of injury after spinal cord contusion or hemisection (Dusart and Schwab, 1994, Carlson et al. 1998, Schnell et al., 1999a). Once activated, most likely through cytokines released by resident CNS cells and/or nearby immune cells that were activated post-traumatically, neutrophils release chemotactic factors such as chemokines, which can attract other inflammatory cells to the site of injury and amplify the immune response. Among the well-documented chemokines secreted by the neutrophils are MIP-1α, MIP-1β, and MCPs (Cassatella 1999, Scapini et al. 2000, Yamashiro et al. 2001), which are all known to recruit granulocytes, lymphocytes and monocytes into inflammatory sites (Ransohoff and Tani 1998, Asensio and Campbell 1999).

A role for activated neutrophils in secondary damage induced after SCI was first suggested several years ago by Means and Anderson (1983). Later, it was shown that neutrophils accumulate in the first few hours following SCI (Blight 1992, Dusart and Schwab 1994, Carlson et al. 1998), and that neutrophil infiltration is proportional to the magnitude of the trauma (Xu et al. 1990, Schoettle et al. 1990). Taoka and colleagues reported that administration of an antibody against P-selectin, a lectin expressed on the endothelial cell surface after injury, attenuated the accumulation of neutrophils in the injured spinal cord and improved motor functions (Taoka et al. 1997). Antibodies directed against intercellular cell-adhesion molecule-1 (ICAM-1) also reduced the infiltration of neutrophils into damaged tissue and reduced the motor loss following spinal cord compression in rats (Hamada et al. 1996a). Blocking the interaction between αDβ2 integrin and vascular cell-adhesion molecule-1 (VCAM-1) with an antibody
directed against αDβ2 reduced the number of neutrophils at the site of SCI by 43% (Mabon et al. 2000). Lacroix and colleagues recently reported that a large number of neutrophils are attracted into the injured spinal cord of rats grafted with fibroblasts releasing a fusion protein consisting of the pro-inflammatory cytokine IL-6 and its alpha receptor which interacts directly with gp130 to transduce the IL-6 signal (Lacroix et al. 2002). This neutrophil influx was associated with increased numbers of macrophages and activated microglia and led to greater tissue damage.

In transgenic mouse models of EAE, increased neutrophil influx was also associated with greater tissue destruction and more severe clinical deficits (Tran et al. 2000). On the other hand, our laboratory has shown that a brief influx of neutrophils into the spinal cord occurring over a period of 6-12 hours after LPC injection into the mouse spinal cord is not detrimental and may contribute to the controlled non-destructive inflammatory response seen in this model (Ousman and David 2000). Taken together, these results suggest that although a limited and brief influx of neutrophils may be required for an effective immune response, excessive neutrophil influx could participate both directly and/or indirectly in secondary tissue destruction after SCI. Neutrophils could accumulate at sites of injury and contribute directly to secondary cell death by secreting cytotoxic factors such as proteases and reactive oxygen species (Harlan 1987). Alternatively, neutrophils could penetrate the CNS and subsequently trigger the leakage of the blood brain barrier via the release of inflammatory mediators. This could stimulate circulatory disturbances and cause the migration of more inflammatory cells to sites of trauma and exacerbate tissue damage.

T lymphocytes:
T cells are essential cellular components of the adaptive immune response. While normally found in very low numbers in the intact brain and spinal cord, T cells rapidly enter the CNS after an injury (Hirschberg and Schwartz, 1995, Popovich et al. 1997) but their numbers in the injured CNS are lower than in injured peripheral nerve, likely due to elimination via cell death (Moalem et al., 1999c). Activated T cells can protect tissue by directly killing foreign pathogens or by orchestrating the humoral immune response. Such a humoral response will lead to the production of specific antibodies and ultimately, the elimination of foreign cells or pathogens via the induction of the complement cascade. However, T lymphocytes are also implicated in autoimmune disease such as multiple sclerosis (MS) and EAE (Traugott et al., 1983, Zamvil et al., 1985).

Despite the evidence that autoimmune T cell responses directed against myelin antigens can cause neural damage in EAE (Traugott et al., 1983, Zamvil et al., 1985), it has been proposed in recent years that inducing autoimmunity via manipulation of autoreactive T cells can contribute to neuroprotection after CNS injury (Schwartz 2001). In the context of SCI, it has been reported that rats with spinal cord contusion injected with myelin basic protein (MBP) reactive T cells show significantly better functional recovery, as measured by the open-field locomotor test of Basso et al., (1995) than rats injected with T cells reactive with ovalbumin
Active immunization with MBP was also shown to promote functional recovery (Hauben et al. 2000). Another study reported that rats with spinal cord contusion injury treated with MBP-activated splenocytes derived from other spinal cord injured rats showed better recovery of hind-limb motor skills than control animals treated with splenocytes derived from naïve recipients (Yoles et al. 2001).

It is not yet clear how autoreactive T lymphocytes exert their protective effect in limiting tissue damage at the site of injury. It has been proposed that neurotrophins released by myelin reactive T cells at sites of injury could mediate some of these effects (Kerschensteiner et al. 1999, Moalem et al. 2000). Brain-derived neurotrophic factor (BDNF) delivered via osmotic minipump (10µg/day) was reported to reduce tissue necrosis and cavity formation, as well as secondary cell death of motor neurons near the lesion site after unilateral spinal cord hemisection in adult rats (Novikova et al., 1996). Effects of other neurotrophins in this regard are not known. Neurotrophins can also be retrogradely transported to the cell soma and enhance the survival of long projecting neurons (Tetzlaff et al., 1994, Tuszynski and Kordower 1999). Whether T cells release sufficient amounts of neurotrophins for an adequate duration of time under the experimental conditions discussed above to mediate such neuronal protection and prevent cavitation has yet to be demonstrated. Other cell types such as macrophages and activated microglia have also been proposed to participate in the neuroprotective effects mediated by myelin reactive T cells (Butovsky et al. 2001).

One shortcoming of using MPB-reactive T cells is that they induce EAE while seemingly being protective (Moalem et al. 1999a, Hauben et al. 2000). The MBP-reactive T cells that are protective in SCI are T helper 1 (Th 1) type T cells, which are also known to underlie the induction of EAE. In an attempt to reduce the probability of inducing EAE, T cells reactive to a potentially non-encephalitogenic cryptic epitope of MBP was tested. Although these T cells were reported to be neuroprotective after optic nerve crush injury, mild EAE paralysis nevertheless occurred in some animals (Moalem et al. 1999a). In other work, Popovich et al. showed that SCI sensitized T cells harvested from rat lymph nodes one week after a contusion injury can induce EAE like symptoms when injected into naïve recipients (Popovich et al. 1996b). However, there are no reports of increased incidence of MS in patients with SCI.

More recently, Schwartz and colleagues showed that a Th 2 T cell response that prevents autoimmune responses also leads to neuroprotection (Kipnis et al. 2000, Hauben et al. 2001). They reported that immunization with MBP in incomplete Freund’s adjuvant, or treatment with copolymer-1 (Cop-1), a non-pathogenic synthetic compound that mimics MBP, also gives protective responses (Kipnis et al. 2000, Hauben et al. 2001). Cop-1 is currently being tested in the treatment of MS. The treatment protocol used with Cop-1 stimulates a Th 2 response, reflected in an increase in IL-10 (Aharoni et al. 2000). Active immunization with Cop-1 or an injection with Cop-1 reactive T cells reduced the death of retinal ganglion cells after a crush injury to the optic nerve (Kipnis et al. 2000). How autoreactive T cells that display a Th 1
phenotype capable of producing autoimmune disease might confer neuroprotection to the injured CNS, while in other instances Th 2 type T cell responses that prevent autoimmune disease do the same is not entirely clear at present.

In other work, Jones and colleagues reported that transgenic mice in which over 95% of all CD4-positive T cells are MBP-reactive had higher intraspinal mRNA levels for pro-inflammatory cytokines, significantly more tissue loss, and impaired motor recovery compared to their littermates after spinal cord trauma (Jones et al. 2002). Notably, these transgenic mice also showed significantly less white matter sparing at sites of injury, and T lymphocytes were colocalized with demyelination. However, 95% myelin reactive T cells are far above the percentage that would be encountered normally after CNS damage. After EAE, for example, it is believed that less than 10% of the infiltrating T lymphocytes are CNS antigen specific (Cross et al. 1990, Steinman 1996). Although the study by Jones et al. (2002) point to the potential detrimental effects of MBP reactive T cells, a potentially protective effect of myelin-reactive T cells at a lower ratio cannot be entirely excluded. Furthermore, there is also the possibility that non-myelin-reactive T lymphocytes, with collaboration from myelin-reactive T cells, could contribute to neuroprotection after CNS injury. In addition, a very short period of T cell influx into the spinal cord of 6-12 hours after LPC injection does not lead to adverse effects, and may be necessary for the subsequent inflammatory changes leading to rapid clearance of myelin damaged by LPC (Ousman and David 2000). The results presented above clearly highlight some of the uncertainties associated at present with manipulating T cells as a therapy for CNS injury. A better understanding of T cell function in the context of CNS pathology after traumatic injury is required before such immunotherapies can be safely applied.

**Macrophages:**

Of all the inflammatory cells, macrophages are present in large number at sites of SCI. A high number of macrophages infiltrate the spinal cord 3 days after spinal cord contusion in rats and mice, and their numbers peak between 7 to 28 days after contusion injury (Popovich et al. 1997; Ma et al., 2002). Depleting macrophages lead to tissue sparing in the injured CNS (Giulian and Robertson 1990, Popovich et al. 1999). Popovich et al. (1999) showed that depletion of blood-derived macrophages using intravenous injections of liposome-encapsulated clodronate decreased cavitation and promoted partial hindlimb recovery after spinal cord contusion. In addition, reduction in the number of macrophages at the site of trauma by blocking αDβ2 integrin with an antibody within 48 hours of a clip compression injury of the spinal cord also reduced lesion size and improved locomotor scores (Gris et al. 2001). That macrophages might exacerbate tissue loss after spinal cord trauma remains, however, a subject of some controversy. When appropriately stimulated macrophages are transplanted into the injured optic nerve or spinal cord, they do not appear to be detrimental to the tissue but instead promote axon repair (Lazarov-Spiegler et al. 1996, Prewitt et al. 1997, Rabchevsky and Streit 1997, Lazarov-Spiegler et al. 1998, Rapalino et al. 1998). Interestingly, macrophages stimulated by incubation *in vitro* with segments of peripheral nerve but not pieces of optic nerve were capable of phagocytosing
myelin as well as stimulating axon regeneration when transplanted into the CNS (Lazarov-Spiegler et al., 1998). Other evidence by Leon et al., (2000) also show that recruitment and activation of macrophages into the eye induces neuroprotection in the retina and regeneration of retinal ganglion cell axons into the lesioned optic nerve. The effects of macrophages on axon regeneration will be dealt with in more detail in the next section. The apparent discrepancies between the harmful and beneficial effects of macrophages may be due at least in part to the differences in the way they are activated, and the time of their activation or transplantation after injury.

Macrophages may produce a different repertoire of cytokines, trophic factors, free radicals, and other molecules depending on how they are activated or their state of activation. Therefore, it is conceivable that macrophages could support tissue repair under certain conditions but under different conditions be detrimental for tissue survival and regeneration. This is clearly illustrated in the two models cited earlier, namely, the EAE and LPC-induced demyelination models. Macrophages induce tissue destruction at sites of EAE lesions (Huitinga et al. 1990, Huitinga et al. 1995, Tran et al. 1998), while after LPC injection into the spinal cord, the activated macrophages strip axons of their damaged myelin sheaths while leaving the axons and neighboring cells intact (Blakemore et al. 1977, Crang and Blakemore 1986, Triarhou and Herndon 1985, Ousman and David 2000, Ousman and David 2001). Differences in these models may be due to the timing and duration of expression of pro-inflammatory chemokines and cytokines and coordinated expression of pro and anti-inflammatory cytokines (Ousman and David, 2001).

A better understanding is needed of how macrophages can be activated to have either tissue protective or damaging properties. Knowledge of the molecular control of such activation and the molecular and functional phenotype of these macrophages is need if we are to effectively manipulate macrophage function to improve the outcome after SCI or other types of CNS injury.

**B. General approaches to modulate the immune response**

*Steroids treatment:*

Anti-inflammatory drugs like methylprednisolone (MP) have been used for several years by physicians to limit the extent of damage after SCI (Young et al. 1994). Although this treatment does not rescue cells directly damaged by the trauma, it is thought to reduce the severity of SCI by reducing secondary cell loss that normally occurs after the initial trauma. Nevertheless, the use of steroids to treat spinal cord injured patients remains controversial.

In a multicenter randomized clinical trial in patients with acute SCI, MP was shown to prevent extensive tissue destruction and to enhance neurological recovery when treatment was initiated within 8 hours after trauma (Bracken et al. 1990). When treatment was initiated early in experimental models of SCI, MP induced long term functional recovery as assessed by locomotor tests (Behrmann et al. 1994). However, in clinical trials (George et al. 1995, Gerndt
et al. 1997, Nesathurai 1998, Hurlbert 2000) and in animal models (Faden et al. 1984, Ross et al. 1993, Haghighi et al. 1998), several other groups have reported a lack of long-term effects of MP on functional recovery after SCI. Takami et al., (2002) reported recently that steroid treatment produced a significant reduction in the volume of damaged tissue in the spinal gray matter without improving axonal sparing or hindlimb locomotor functions after spinal cord contusion. However, sterological analysis by Rabchevsky et al., (2002) indicate that MP treatment had only a marginal effect on lesion volume. They also reported that MP had no effect on the amount of sparing of gray and white matter and failed to improve hind limb function. If MP has any effect on reducing the lesion volume and cavitation, this may be of value, as it would enhance efforts to promote axon regeneration across the lesion.

In addition to any of the possible effects of systemically administered steroids in potentially reducing tissue damage at the site of CNS injury, our laboratory has also studied the effects of topical applications of glucocorticoids on stab wounds to the cerebral cortex in adult rats (Li and David 1996). This study showed that topically applied steroids produce a remarkable reduction in the astroglial/fibrotic scar and the formation of the glia limitans at the site of cortical stab wounds. This effect is due in part to the influence of the steroids on the proliferation and migration of leptomeningeal cells (Li and David, 1996), which is necessary for the formation of the glia limitans (Sievers et al., 1994). The mechanism of action is analogous to the effects of steroids in reducing keloid formation during wound healing of the skin (William and Mertz, 1978). Therefore, steroids can influence both the level of tissue damage at the site of SCI trauma as well as reduce the development of the astroglial/fibroblastic scar.

Cyclooxygenase-2 inhibitors:
Over the last few years, it has been suggested that prostaglandins might play a role in inflammation and secondary injury in various tissues and organs. Of particular interest are recent studies showing that blocking the synthesis of prostaglandins using non-steroidal anti-inflammatory drugs prevents neuronal cell death after various types of CNS insults (Patel et al. 1993, Nogawa et al. 1997, Nakayama et al. 1998, Kunz and Oliw 2001). Significantly higher levels of eicosanoids are present in the cerebrospinal fluid and at the site of lesion after SCI (Mitsuhashi et al. 1994, Nishisho et al. 1996, Tonai et al. 1999). The mRNA and protein levels of cyclooxygenase (COX)-1 and -2, the rate-limiting enzymes for the conversion of arachidonic acid into prostaglandins, are also increased in the injured spinal cord (Resnick et al. 1998, Schwab et al. 2000).

Recent work indicates that COX-1 is involved in gastrointestinal and renal homeostasis, whereas COX-2 is involved in inflammation and might be responsible for most of the prostaglandin-mediated cytotoxic effects (Smith and Dewitt 1996). Interestingly, COX-2 is the predominant COX isoform expressed in the CNS (Lacroix and Rivest 1998). After an immune insult or treatment with pro-inflammatory cytokines, COX-2 is expressed in monocytes and throughout the brain vasculature (Lacroix and Rivest 1998, Schiltz and Sawchenko 2002).
Selective COX-2 inhibitors have been tested in neuroprotection studies (Nogawa et al. 1997, Nakayama et al. 1998). Notably, administration of the selective COX-2 inhibitor NS-398 reduced tissue loss, nociceptive behavior, and locomotor deficits after spinal cord contusion in rats (Hains et al. 2001). Although there is a correlation between tissue preservation and the reduction of inflammatory cells at sites of injury, it is possible that blocking the synthesis of prostaglandins might confer neuroprotection by preventing a post-traumatic decrease in spinal cord blood flow and ischemia (Hall and Wolf 1986). Therefore, there is a need to clarify the mechanism of action of COX-2 inhibitors in the injured spinal cord, optimize the dosage, and define the therapeutic window. Furthermore, based on at least two studies, which suggest that multiple injections of COX inhibitor either have no effect or exacerbate tissue loss and motor deficits after CNS trauma (Guth et al. 1994, Dash et al. 2000), the effects of single versus multiple treatments will need to be compared. One possible explanation for these results could be that COX-2 might have pro-inflammatory effects during the early phase of inflammation but might help resolve the inflammatory response at later stages, as was recently suggested using a model of carrageenin-induced pleurisy (Gilroy et al. 1999).

Investigations using COX-2 knockout mice have also raised important questions that need to be answered. These studies have demonstrated that COX-2 is essential for renal and cardiac homeostasis and the attenuation of the inflammatory reaction in response to several experimental models of inflammation (Morham et al. 1995, Dinchuk et al. 1995, Wallace et al. 1998, Gilroy et al. 1999, Komhoff et al. 2000). Notably, COX-2-deficient mice had a considerably reduced life span compared to wild type mice (Morham et al. 1995). Other studies have reported that COX-1 can display pro-inflammatory properties, which vary depending on the organ and on the stage of the inflammatory response (Langenbach et al. 1995, Wallace et al. 1998). In the CNS, Schwab and colleagues have recently reported that COX-1 expression is induced in macrophages and activated microglia at the site of cortical stab wounds or SCI (Schwab et al. 2000, Schwab et al. 2001). These results suggest that COX-1 could also be involved in inflammatory processes and secondary damage following CNS trauma. A better understanding of the contribution of COX enzymes to the pathology seen after CNS trauma is needed before treatments can be applied safely.

7.3. Stimulating axon regeneration
Although axon regeneration does not occur in the damaged adult mammalian CNS, earlier work by David and Aguayo showed that mature CNS neurons retain the ability to regenerate for long distances if they are provided with an appropriate glial environment (David and Aguayo 1981). Multiple factors underlie the failure of axon regeneration through CNS tissue. However, work done in the past two decades has revealed that molecules that inhibit axon regeneration play a particularly important role. These inhibitors of axon growth are found in the glial scar and in myelin (David 1998).

7.3.1. Immune involvement in the formation of the glial scar
Cytokines such as IL-1β, interferon-gamma (IFNγ), TGFβ, and TNFα produced by immune cells are known to stimulate astrogliosis and the formation of the glial scar (Yong et al. 1991, Feuerstein et al. 1994, Balasingam et al. 1994, Lagord et al., 2002). Immune cells that enter the site of CNS contusion injury could therefore be sources of these cytokines and contribute to the development of the scar. Furthermore, astrocytes are also capable of secreting chemokines and cytokines such as MCP-1, MIP-1α, IL-1β and TNFα (Hurwitz et al. 1995, Guo et al. 1998, Ransohoff and Tani 1998, Asensio and Campbell 1999) that can regulate the influx of immune cells into the CNS parenchyma and lead to their activation. Immune cells and astrocytes are therefore capable of signaling each other, and this may have the effect of prolonging the immune cell response locally at the site of CNS injury as well as leading to the glial scar. Blunting the immune response or neutralizing chemokines or cytokines at the site of injury may therefore be one approach to limiting the extent of gliosis.

Reactive astrocytes that form the scar secrete chondroitin sulfate proteoglycans that can inhibit neurite growth in vitro (McKeon et al. 1991) and axon growth in vivo (Davies et al. 1997, Davies et al. 1999). Recent studies have shown that the inhibitory effects of the glial scar can be neutralized with the enzyme chondroitinase ABC. Unlike other methods to modulate the scar, treatment with this enzyme promotes axon regeneration in the injured brainstem and spinal cord (Moon et al. 2001, Bradbury et al. 2002). As this method does not involve the immune response it will not be dealt with further in this review.

### 7.3.2. Inhibition of axon growth by myelin

In addition to the glial scar, myelin also has very potent neurite and axon growth inhibitory activity (Caroni and Schwab 1988, David, 1998, Bandtlow and Schwab 2000). Unlike the glial scar, which forms in response to injury and takes a few days to be fully established, myelin is always present in the CNS and is therefore in a position to immediately exert its inhibitory effects and prevent axon regeneration. Neutralizing the axon growth inhibitory effects of myelin is therefore one of the prime approaches to foster axon regeneration after SCI. Work done over the past decade and a half has revealed that there are several inhibitory activities in myelin, three of which have been identified and characterized. These inhibitors are Nogo-A, myelin-associated glycoprotein (MAG) and the recently identified inhibitor oligodendrocyte myelin glycoprotein (OMgp).

Nogo-A is a splice variant of Nogo, a glycoprotein that belongs to the reticulon family (Chen et al. 2000, GrandPre et al. 2000, Prinjha et al. 2000). It was first identified by Caroni and Schwab (1988) using the monoclonal antibody IN-1. Numerous studies over the past 15 years have detailed the ability of this molecule to inhibit axon growth in vitro and in vivo (Huber and Schwab 2000, Fouad et al. 2001). Two inhibitory domains have been identified in Nogo-A, one of which is a 66-amino acid region (GrandPre et al. 2000). The receptor to this region (Nogo-66 receptor) has been isolated (Fournier et al. 2001). The neurite growth inhibitory effect of MAG was first described by Mukhopadhyay et al. (1994) and McKerracher et al. (1994). The
potent neurite growth inhibitory effects of MAG have also been well characterized (Li et al. 1996, Shibata et al. 1998, David and McKerracher 1999). Recent evidence indicates that MAG mediates inhibition via the Nogo receptor and competes with Nogo-66 for the same binding site (Domeniconi et al. 2002, Liu et al. 2002). The inhibitor OMgp is a glycosylphosphatidylinositol-linked glycoprotein that like Nogo-A and MAG induces growth cone collapse and inhibits neurite growth. Surprisingly, OMgp also mediates its inhibitory effects on growth cones via the Nogo receptor but interacts with a different binding site than Nogo-66 (Wang et al. 2002).

7.3.3. Neutralizing axon growth inhibitors in myelin

All three axon growth inhibitors in myelin bind to the same receptor on the growth cone to inhibit neurite growth. Recent studies have revealed that neurite growth inhibition induced by myelin substrates is mediated via cAMP and the small GTPase Rho-dependent mechanisms (David and Lacroix, 2003). Axon growth on myelin substrates in vitro can be promoted by increasing intracellular cAMP levels (Ming et al. 1997, Song et al. 1998, Cai et al. 1999, Cai et al. 2001) or inactivating Rho (Lehmann et al. 1999, Winton et al. 2002). These treatments have also been reported to have some effect in promoting axon regeneration in the spinal cord in vivo (Lehmann et al. 1999, Qiu et al. 2002, Neumann et al. 2002, Dergham et al. 2002). Here we will focus attention on two other approaches to neutralize these inhibitors that involve the immune system. One approach is to block the inhibitors with function-blocking antibodies. The other approach is to eliminate or reduce the axon growth inhibitory effects of myelin by rapidly clearing the myelin from areas of the CNS that are damaged and undergoing Wallerian degeneration.

A. Neutralizing axon growth inhibitors in myelin with antibodies

Use of Monoclonal antibodies:
The first immunological tool that was used to neutralize axon growth inhibitors in myelin was the monoclonal antibody IN-1 (Caroni and Schwab 1988). These in vitro studies were soon followed by the in vivo demonstration that delivery of this monoclonal antibody via the transplantation of the hybridoma cells into the brain led to long distance axon regeneration in the injured adult rat spinal cord (Schnell and Schwab 1990). Martin Schwab’s group has shown that this monoclonal antibody is able to promote regeneration of axons in various regions of the adult mammalian CNS (Bandtlow and Schwab, 2000). A large body of evidence now indicates that treatment with this monoclonal antibody leads to regeneration of damaged axons, as well as sprouting of intact fibers from the contralateral tract or adjacent tracts that then reinnervate the denervated targets (Bandtlow and Schwab 2000, Raineteau and Schwab 2001a,b). Partially humanized recombinant Fab fragments of the IN-1 antibody delivered by osmotic pump to the site of spinal cord hemisection was also able to promote long distance axon regeneration (Brosamle et al. 2000). The monoclonal antibody IN-1 recognizes only one of the inhibitors in myelin. This leaves other inhibitors to continue to exert their effect and may explain why only a small proportion of the damaged fibers regenerate after treatment with this antibody. To block
all of the inhibitors with the monoclonal antibody approach will require using a cocktail of monoclonal antibodies against each of the inhibitors or their receptor. This will have to be tested in the future. The delivery of monoclonal antibodies is not a method that directly co-opts the immune response to promote regeneration.

*Use of a vaccine approach:*
We have tested a method that has the potential to block multiple axon growth inhibitors in myelin by co-opting the animal’s own immune system to generate function-blocking antibodies (Huang *et al.* 1999). This is essentially a therapeutic vaccine approach using myelin as the immunogen. In an effort to prevent the development of autoimmune disease against myelin antigens, i.e., the generation of EAE, mice were immunized with myelin in incomplete Freund’s adjuvant (IFA). EAE is primarily a T cell mediated disease. An acute episode of EAE is provoked by a Th1 T cell response, which produces pro-inflammatory cytokines while a Th2 response is protective and present during remission. Immunizing mice with myelin or spinal cord homogenates in IFA prevents the development of EAE (Rodriguez *et al.* 1987, Rodriguez 1991, O’Neill *et al.* 1992, Rivero *et al.* 1997). Such myelin antigen induced tolerance and prevention of autoimmune disease may occur via a number of possible mechanisms such as T cell anergy, reduced responsiveness of antigen-specific T cells, action of suppressor cells and/or switch from Th 1 to Th 2 cells (Gaur *et al.* 1992, O’Neill *et al.* 1992, Weiner *et al.* 1994, Marusic and Tonegawa 1997). Immunization with myelin or spinal cord homogenate in IFA also induces the production of anti-myelin antibodies (Rodriguez *et al.* 1987, Rodriguez 1991, Rivero *et al.* 1997). One monoclonal antibody generated by such immunizations is capable of inducing remyelination in animal models of demyelination (Rodriguez *et al.* 1987, Rodriguez 1991).

The question we addressed is whether immunization with myelin in IFA will result in the generation of antibodies capable of neutralizing the inhibitors in myelin and promote long distance axon regeneration. One important determinant of the success of this approach is whether the antibodies in the circulation are able to cross the blood-brain barrier. Our work shows that antibodies that are generated are able to block the neurite growth inhibitory effects of myelin. In addition, the antibodies in the circulation were found to cross the blood-brain barrier and bind to myelin in the myelinated tracts adjacent to the lesion that were damaged by the spinal cord hemisection. Other studies have shown that the blood-brain barrier becomes permeable for a distance of 25 mm after spinal cord contusion (Popovich *et al.* 1996a). Additionally, the blood-brain barrier also becomes leaky in CNS pathways undergoing Wallerian degeneration likely through mechanisms triggered by activated macrophages (Jensen *et al.* 1997). In our immunization experiments, the spinal cord lesions that were made were small so as to produce a minimal glial scar. Under these conditions, immunization with myelin resulted in robust axon regeneration for distances of up to 11 mm, which is about 1/4 the length of the mouse spinal cord (Huang *et al.* 1999). In addition to the antibody-mediated effects, the vaccine approach has also been shown to stimulate increased macrophage activation and myelin clearance (Kuo *et al*., 2001, Avilés-Trigueros and David, unpublished observations). This effect
of the immunization may contribute further to the reduction of the myelin-associated inhibitory activity and the promotion of axon regeneration.

These experiments indicate that, in principle, an immunization strategy could be employed to block myelin-associated inhibitors of axon growth to promote axon regeneration after SCI. Although we were able to prevent EAE in an inbred mouse strain while promoting axon regeneration, it may be difficult to ensure complete protection from an autoimmune response in humans. A safer alternative for the present may be to use a cocktail of purified inhibitors as the immunogen after ensuring that the inhibitory molecules themselves are not encephalitogenic. Our recent studies on SJL/J mice immunized with recombinant Nogo-66 and MAG in alum indicate that some degree of axon regeneration can be stimulated without inducing EAE (Sicotte et al., 2003).

B. Neutralizing the inhibitory effects of myelin by stimulating rapid myelin phagocytosis
Stimulating myelin phagocytosis may be another way to rid the affected regions of the CNS of the inhibitory effects of myelin and promote axon regeneration. This appears to be the method that peripheral nerves employ to create an environment that is conducive for axon regeneration. Peripheral nerves regenerate robustly, even though peripheral nerve myelin is as inhibitory as CNS myelin (David et al. 1995). This difference in the capacity for regeneration in the CNS and peripheral nerves may be due to differences in the immune response. Immune cells enter damaged peripheral nerves undergoing Wallerian degeneration within 1-3 days after lesion, and phagocytose myelin and axonal debris within 3–10 days (Bruck 1997). In contrast, immune activation and myelin clearance from CNS regions undergoing Wallerian degeneration is extremely slow, taking several weeks to months in rodents, and years in humans (George and Griffin, 1994, Perry et al. 1987, Miklossy and Van der Loos 1991). As a result, the inhibitors in CNS myelin can continue to exert their inhibitory effects on axon regeneration for prolonged periods of time after injury.

David et al. (1990) provided the first direct evidence that activated macrophages can reduce the inhibitory properties of myelinated regions of the CNS. This study showed that myelinated rat optic nerve sections treated with activated macrophages harvested from a cerebral cortical stab wound or peritoneal macrophages activated with muramyl dipeptide were able to convert the inhibitory optic nerve to a permissive substrate for neurite growth. In addition to the ability of macrophages to phagocytose myelin from damaged areas of the CNS, this in vitro study showed that macrophages also secrete molecules capable of neutralizing the inhibitors in myelin. Subsequently, several other groups extended these findings to in vivo studies to show that injection of activated macrophages into the adult rat optic nerve or spinal cord results in promotion of axon regeneration after injury (Lazarov-Spiegler et al. 1996, Prewitt et al. 1997, Rabchevsky and Streit 1997, Lazarov-Spiegler et al. 1998, Rapalino et al. 1998). It has been suggested that an inhibitory factor in the CNS blocks macrophage recruitment into the injured CNS (Hirschberg and Schwartz, 1995). However, our more recent studies indicated that, under
appropriate experimental conditions, hematogenous macrophages can be recruited rapidly into the CNS and activated along with resident microglia to phagocytose myelin from white matter (Ousman and David 2000; Ousman and David 2001).

An alternative approach to macrophage transplantation is to trigger rapid macrophage influx and activation directly into damaged regions of the CNS or regions undergoing Wallerian degeneration. To achieve this will require the identification of the immune modulators, namely the chemokines and cytokines that regulate rapid myelin clearance from the CNS. This macrophage activation will have to be achieved without provoking a detrimental inflammatory response that leads to secondary tissue damage such as occurs in EAE. In an effort to identify such molecules we utilized the LPC-induced demyelination model in which macrophage activation and myelin clearance occurs without associated damage to axons or neural cells. This work led to the identification of MCP-1, MIP-1α, granulocyte macrophage-colony stimulating factor (GM-CSF), and TNFα as being key players in regulating the immune response, i.e., T cell and monocyte/macrophage influx into CNS parenchyma and macrophage activation leading to rapid clearance of damaged myelin without destruction of adjacent cells (Ousman and David 2001). It will be of interest to know if these or other cytokines (Shamash et al. 2002) and chemokines can enhance Wallerian degeneration in the CNS and if they can be employed to promote rapid clearance of myelin from such areas of the CNS after injury to promote axon regeneration.

7.4. Concluding comments
In the past decade we have learned a great deal about the nature of the immune cell response after SCI and the associated changes in chemokines and cytokines. This has led to the testing of a variety of approaches to either block the entry of immune cells into the CNS to reduce tissue damage or the activation of some of these cell types to promote neuroprotective and regenerative effects. As we begin to understand the molecular mechanisms that regulate the activation of these cells toward different phenotypes, and the molecular nature of these phenotypes we will be able to make sense of the seemingly contradictory evidence we now have with regards to the role of macrophages and T cells in SCI. It may then be possible to develop effective approaches to treat SCI by regulating the recruitment and activation of these immune cells.

We can also expect further development of antibody approaches to neutralize axon growth inhibitors. The success of such approaches will require a better understanding of B cell activation, of adjuvants, and of autoimmune responses and ways to control them. Furthermore, understanding the molecular control of Wallerian degeneration will also permit the development of novel strategies to clear myelin rapidly from areas of the injured CNS and create an environment that is permissive for regeneration.
References


