

THE ROLE OF COMPLEMENT IN THE IMMUNOLOGICAL DEMYELINATION  
OF THE ADULT RAT SPINAL CORD

By

JASON ALAIN BOURQUE

B.Sc., University of British Columbia, 1995

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES  
(DEPARTMENT OF ZOOLOGY)

We accept this thesis as conforming  
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 2000

© Jason Alain Bourque, 2000

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of ZOOLOGY

The University of British Columbia  
Vancouver, Canada

Date AUGUST 18, 2000

## ABSTRACT

Previous work in Dr. John Steeves' laboratory has described a transient, myelin-specific, galactocerebroside (GalC) antibody-mediated, complement-dependent protocol that suppresses the onset of myelination in embryonic birds, and focally demyelinate the central nervous system (CNS) of adult birds and rodents after intraspinal infusion. After spinal injury, this procedure has been observed to facilitate axonal regeneration, often with accompanying recovery of behavior. In order to identify the relative necessity of different complement proteins in this protocol, I assessed the effects of infusions of GalC-antibody and human serum deficient for a particular complement protein (i.e. C3, C4, C5, or Factor B was immunoabsorbed prior to infusion) on spinal myelin. Spinal treated tissue was evaluated ultrastructurally using a transmission electron microscope. Upon removal of the C3 protein, common to both the classical and alternative complement pathways, or the C4 protein, a classical pathway protein, normal myelin was observed (i.e. no demyelination). Using serum deficient in Factor B, an alternative pathway protein, demyelination was still observed; in addition, large numbers of macrophages containing myelin debris were present in these regions. These results suggest that the classical serum complement pathway has a fundamental role in this immunological demyelination protocol. Upon removal of C5, a Membrane Attack Complex (MAC) protein, substantial demyelination was again observed, once more accompanied by macrophages, suggesting that some of the demyelination may be occurring through macrophage-mediated events involving complement-derived anaphylatoxins and membrane receptors. Taken together, these findings suggest that the classical pathway of complement activation and anti-GalC IgG antibody are sufficient,

and thus the alternative pathway and the MAC are not necessary, for focal demyelination of the adult rat spinal cord, and that this demyelination occurs in a macrophage-dependent manner.

## TABLE OF CONTENTS

Abstract	ii
Table of Contents	iv
List of Figures	v
List of Abbreviations	vi
CHAPTER 1) INTRODUCTION	1
1.1) Myelin in the CNS	2
1.2) Demyelination	9
1.3) The Complement Cascade	13
<i>1.3a) Introduction</i>	13
<i>1.3b) The Classical Pathway</i>	14
<i>1.3c) The Alternative Pathway</i>	19
<i>1.3d) The Membrane Attack Complex</i>	23
<i>1.3e) Inflammation and Anaphylaxis</i>	26
1.4) Complement and the Nervous System	27
1.5) Complement In Host Defense and Disease	29
1.6) Hypothesis and Experimental Plan	32
CHAPTER 2) MATERIALS AND METHODS	33
2.1) Choice of Depleted Sera	34
2.2) Antibody Production	34
2.3) Transient Immunological Myelin Disruption	35
2.3) Electron Microscopy	36
CHAPTER 3) RESULTS	41
CHAPTER 4) DISCUSSION	56
REFERENCES	77

**LIST OF FIGURES**

Figure 1)	The classical pathway of complement activation.	16
Figure 2)	The alternative pathway of complement activation.	20
Figure 3)	The membrane attack complex.	24
Figure 4)	The complement cascade with experimental deficiency points.	38
Figure 5)	Complete human complement and anti-GalC IgG infusions compared to controls of complement or antibody alone.	47
Figure 6)	Classical and alternative pathway contributions to demyelination.	50
Figure 7)	C3 and macrophage contributions to demyelination.	52
Figure 8)	Membrane attack complex contribution to demyelination.	54

**LIST OF ABBREVIATIONS**

BBB	Blood-brain barrier
C	Complement protein
CNP	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CR	Complement receptor
CSF	Cerebrospinal fluid
DRG	Dorsal root ganglia
E	Embryonic day
EAE	Experimental allergic encephalomyelitis
ECM	Extracellular matrix
GalC	Galactocerebroside
GBS	Guillain-Barré syndrome
Ig	Immunoglobulin
IL	Interleukin
MAC	Membrane attack complex
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MS	Multiple sclerosis
Omgp	Oligodendrocyte-myelin glycoprotein
PLP	Proteolipid protein
PNS	Peripheral nervous system
T	Thoracic
TMEV	Theiler's murine encephalomyelitis virus
TNF	Tumour necrosis factor

**CHAPTER 1**

**INTRODUCTION**

### 1.1) Myelin in the CNS

The adult mammalian central nervous system (CNS) is restricted in its ability to support the regeneration of injured axons. Damage to adult CNS axons leads to degeneration of the severed axons after aborted attempts at regeneration (Bjorklund et al., 1971; Ramon Y Cajal, 1928), and a subsequent slower degeneration of the surrounding myelin (Waller, 1850). However, these neurons have been shown to possess the intrinsic capacity to regenerate if certain conditions exist. CNS axons will regrow if allowed to project through a permissive environment, such as a peripheral nerve graft (Cheng et al., 1996; David and Aguayo, 1981; Richardson et al., 1980; Tello, 1911), but with very limited regrowth back into the CNS environment. CNS axonal growth can also be increased by means of Schwann cell injections or implants (Li and Raisman, 1994; Paino et al., 1994; Xu et al., 1995), olfactory ensheathing cell transplants (Li et al., 1997; Ramon-Cueto et al., 2000), and fetal graft transplants (Bregman et al., 1989) into adult CNS lesions. These findings suggest that the CNS environment itself may possess properties inhibitory for axonal growth.

It has been suggested that one of the principal barriers to CNS axonal regeneration is the presence of inhibitory growth molecules. Collapsin (Luo et al., 1993) and other members of the semaphorin family (Kolodkin et al., 1993) have been shown to induce growth cone collapse. Tenascin is a secreted extracellular matrix (ECM) component that is expressed in the region of the glial scar after injury (Laywell et al., 1992) and has accounted for both promotion and inhibition of neurite outgrowth, depending on the isoform expressed (Ajemian et al., 1994; Fruttiger et al., 1995; Gotz et al., 1996; McKeon et al., 1991). Janusin/J1-160 is a tenascin-related molecule produced

by oligodendrocytes (Pesheva et al., 1989) that repulses growth cones (Taylor et al., 1993). In addition, chondroitin sulfate and keratan sulfate proteoglycans are ECM proteins that are localized within the glial scar (Dow et al., 1994; Levine, 1994; McKeon et al., 1991) as well as sites of axonal boundaries in the developing CNS (Pindzola et al., 1993) and have inhibitory effects on growth cones *in vitro* (McKeon et al., 1991; Oohira et al., 1991; Snow et al., 1990). Most of these inhibitory molecules are associated with the glial scar that forms after spinal cord injury.

CNS myelin has also been shown to have strong inhibitory activity for axonal growth. Myelin is a highly-organized multilamellar structure that surrounds neuronal axons, and is formed by the plasma membrane of oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system (PNS) (for review see Morell et al., 1994). This specialized membrane acts to facilitate the conduction of action potentials by providing axonal insulation and clustering sodium channels into the nodes of Ranvier, evolutionary developments which were crucial to the formation of the complex nervous systems seen in vertebrates. The importance of myelin in the normal functioning of the CNS is demonstrated in the deficits produced in the many disease states associated with the loss or alteration of normal myelin, such as multiple sclerosis (MS), Pelizaeus-Merzbacher disease, and Charcot-Marie-Tooth disease. Myelination within the CNS usually occurs after the developing axons have made connections with their targets (Jhaveri et al., 1992; Schreyer and Jones, 1982). Therefore, one further possible role of myelin could be to stabilize these connections by inhibiting further growth of axons (Kapfhammer and Schwab, 1994).

Myelin consists of a number of proteins and lipids that are integrated specifically into a very stable and uniform structure (for review of myelin structure see Quarles et al., 1997). Proteolipid protein (PLP) (50% of the total protein of isolated myelin) and P0 (60%) are the major integral myelin membrane proteins of the CNS and PNS, respectively. Both CNS and PNS myelin contain myelin basic protein (MBP) (35% CNS, 10% PNS) and P2 (<1%) which associate with the cytoplasmic sides of the layered membranes. PLP and MBP are thought to stabilize the intraperiod and major dense lines of compact CNS myelin, respectively.

Other proteins associated with CNS myelin include myelin-associated glycoprotein (MAG) (1%), myelin-oligodendrocyte glycoprotein (MOG) (0.1%), and oligodendrocyte-myelin glycoprotein (Omgp), which may be involved in recognition, adhesion, and cellular signalling. The enzyme 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (5%) associates with myelin (Trapp et al., 1988), and may have cytoskeletal functions and act in the formation and maintenance of myelin.

Approximately 70% of the dry weight of myelin is lipid, with the galactolipid, galactocerebroside (GalC), and its sulfated derivative, sulfatide, encompassing about one-third of the lipid mass (Norton and Cammer, 1984). These galactolipids occupy the extracellular face of the membrane. Several *in vitro* studies have suggested that these galactolipids transduce developmental signals (Bansal and Pfeiffer, 1989), facilitate protein trafficking (Brown and Rose, 1992; Kramer et al., 1997; Simons and Ikonen, 1997), and stabilize membranes (Koshy and Boggs, 1996; Koynova and Caffrey, 1995). In addition, mice lacking the ability to synthesize GalC or sulfatide form dysfunctional and unstable myelin (Bosio et al., 1996; Coetzee et al., 1996), suggesting that the

galactolipids, together with the myelin proteins, contribute significantly to the functional and structural properties of myelin. Examination of the structure and function of these various myelin components has helped elucidate the functions of the myelin membrane itself.

Several examples in nature indicate that the presence of CNS myelin may inhibit the growth of CNS axons after injury. Lesioned fiber tracts in the spinal cord of invertebrates and lower vertebrates demonstrate significant regeneration. Lampreys show extensive regeneration and full behavioral recovery within a few months after complete spinal cord transection at both larval and adult stages (Cohen et al., 1988; Davis and McClellan, 1994a; Davis and McClellan, 1994b; Lurie et al., 1994; Lurie and Selzer, 1991). There is also a high degree of anatomical regeneration of descending neurons and functional recovery following the transection of goldfish spinal cord (Bernstein, 1967; Grant and Keating, 1986; Sharma et al., 1993). Salamanders recover swimming function and show regeneration of axons following complete spinal cord transection (Clarke et al., 1988; Davis et al., 1990; Davis et al., 1989). In contrast, frogs only exhibit spinal cord regeneration at the larval stage, but not as adults (Beattie et al., 1990; Forehand and Farel, 1982). It is important to note that significant differences exist between the myelin and oligodendrocytes of lower vertebrates compared to those of higher vertebrates, possibly facilitating the regeneration observed in lower vertebrates.

The CNS of lower vertebrates lacks either myelin itself or some of myelin's functional components. For example, lampreys lack compact myelin at all stages of development. Several of the major myelin components of fish and premetamorphic amphibians are different than those of higher vertebrates (Waehneltdt et al., 1986), and

myelin from fish has lower levels of growth inhibitory proteins and is less inhibitory than myelin from rat spinal cord, (Bastmeyer et al., 1991; Sivron et al., 1994). In frogs, inhibitory myelin antigens are only expressed after metamorphosis (Lang et al., 1995). Furthermore, removal of myelin debris by macrophages is much slower in higher vertebrates compared to lower invertebrates, so injured axons of higher vertebrates may be exposed to material inhibitory to axonal growth for a longer period of time (Dowding et al., 1991; Perry et al., 1993; Perry et al., 1987). It has been suggested that the differences between lower and higher vertebrate myelin could be the basis for the loss of axonal regenerative ability in the CNS of higher animals.

In higher vertebrates, the loss of the ability to regenerate lesioned axons is age-dependent. Newborn hamsters demonstrate extensive corticospinal fiber growth around lesion sites (Kalil and Reh, 1982), and some functional improvement is demonstrated (Reh and Kalil, 1982), although it is not restored to normal levels (Keifer and Kalil, 1991). Similar results were found in newborn kittens (Tolbert and Der, 1987) and in newborn rats (Schreyer and Jones, 1983), in which it was found that the number of axons growing down the spinal cord after lesion decreased with increasing postnatal age (Bates and Stelzner, 1993). In contrast, adult cats (Bregman et al., 1995), hamsters (Keifer and Kalil, 1991), and rats (Schnell and Schwab, 1993), show no extension of axons beyond the lesioned corticospinal tract. This transition may be due in part to the appearance of myelin, an early-postnatal event in mammals.

Studies have been done to determine the transitional developmental stage at which spinal cord regeneration changes from a permissive to a restrictive state and how it may relate to the presence of myelin in some of the higher vertebrates described above.

In the spinal cord of chick embryo, myelin forms on E13. Complete spinal cord transections done prior to E13 led to growth of fibers across the lesion site and functional recovery (Hasan et al., 1993; Shimizu et al., 1990). However, coincident with the onset of myelination, transections on or after E13 resulted in a decrease in both fiber growth across the lesion and in functional recovery. Similar results were found in experiments with early postnatal opossum (Treherne et al., 1992; Varga et al., 1995a; Xu and Martin, 1989; Xu and Martin, 1991) and fetal rat CNS (Saunders et al., 1992), in which extensive growth of axons was observed in these animals, but not in adults. As in the chick, the end of the permissive period for spinal cord repair in opossum and rat correlates with the start of myelin formation in these animals.

It has long been recognized that adult mammalian CNS myelin is inhibitory for axonal regeneration, whereas PNS myelin is not (Berry, 1982). Recently, however, axonal growth inhibitory properties have been attributed to specific myelin-associated molecules. Initial studies showed that oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth *in vitro*. One inhibitory component present in the CNS, but not PNS, is recognized by the monoclonal antibody IN-1 (Caroni and Schwab, 1988b; Caroni and Schwab, 1988c), which was raised against two protein fractions of myelin that strongly inhibit axonal growth. These protein fractions were initially known as NI-35/250, but have recently been renamed Nogo (Chen et al., 2000). This antibody neutralized the nonpermissive substrate properties of the CNS white matter *in vitro* (Caroni et al., 1988a; Caroni and Schwab, 1988b; Caroni and Schwab, 1988c; Crutcher, 1989; Savio and Schwab, 1990; Schwab and Caroni, 1988). *In vivo* application of IN-1 in the rat spinal cord facilitates the regeneration of a variety of lesioned fiber

tracts in rats, including the corticospinal tract (Bregman et al., 1995; Schnell and Schwab, 1990; Schnell and Schwab, 1993), the optic nerve (Weibel et al., 1994), and the cholinergic septohippocampal pathway (Cadelli and Schwab, 1991). Nogo-related activity is found in CNS myelin of other higher vertebrates such as opossum (Varga et al., 1995b), bovine (Spillmann et al., 1998), and human (Spillmann et al., 1997), but not in fish (Caroni and Schwab, 1988c; Wanner et al., 1995), possibly explaining the regenerative capacity of fish axons as alluded to above.

A neurite growth inhibitory effect has also been attributed to the myelin-associated glycoprotein (MAG), present in both CNS and PNS myelin. Using neuroblastoma cells, McKerracher et al (1994) found a major peak of inhibitory activity corresponding to MAG, and the immunodepletion of MAG removed the neurite growth inhibition. Mukhopadhyay et al (1994) demonstrated that MAG strongly inhibits neurite outgrowth from both developing cerebellar and dorsal root ganglion neurons *in vitro*, and this inhibition is reversed upon application of anti-MAG antibody. In contrast to these findings, MAG has also been shown to promote neurite outgrowth of newborn dorsal root ganglia (DRG) cells (Mukhopadhyay et al., 1994) and to promote adhesion of oligodendrocytes and neurons (Poltorak et al., 1987), thereby leading to the classification of MAG as a bifunctional molecule.

Further study of the inhibitory effects of myelin and its particular components to axonal regeneration will most likely lead to the discovery of other proteins with inhibitory activity, making the selective targeting of the individual components in attempts to remove the inhibition ineffective.

## 1.2) Demyelination

Since it is evident that several myelin-associated molecules such as Nogo and MAG demonstrate significant inhibitory activity to neuronal regeneration, it may be advantageous to develop and utilize techniques that will focally and transiently remove whole myelin, rather than one of the several independent molecules, in any attempts to facilitate significant axonal regeneration. Several such experimental demyelination protocols have been used in the past.

One approach to remove myelin from the spinal cord is X-ray irradiation, which interferes with mitosis, thus killing all actively dividing cells, including oligodendrocytes, but not the postmitotic neurons (Blakemore, 1977). When applied to the adult rat spinal cord, glia are targeted, resulting in the demyelination of the adult spinal cord through oligodendrocyte death. Subsequent remyelination of the cord occurs by invading Schwann cells (Sims et al., 1985).

Viruses such as Theiler's murine encephalomyelitis virus (TMEV) and Epstein-Barr virus are also known to trigger demyelination (Dal Canto and Lipton, 1980). However, these models are known to result in oligodendrocyte death and extensive inflammation, which may cause axonal degeneration (Dal Canto and Lipton, 1980). Viral infection is also very unpredictable at lesion sites and could cause host death. Furthermore, TMEV infection is quite prolonged (~ 8 months) and remyelination is predominantly due to Schwann cells (Dal Canto and Lipton, 1980).

Demyelination can also be achieved through the administration of drugs such as lysolecithin (Blakemore, 1978), cuprizone (Ludwin, 1978), ethidium bromide (Graca and Blakemore, 1986; Yajima and Suzuki, 1979), 6-aminonicotinamide (Blakemore, 1975),

and diphtheria toxin (Eames et al., 1977). Unfortunately, variable results and unwanted side effects can accompany demyelination induced by such drugs. For example, lysolecithin is known to attack all membranous cells, has adverse effects on astrocytes, has relatively long-term effects (6 months), and remyelination following treatment is often accomplished by Schwann cells. Cuprizone induces nonfocal demyelination and promotes the proliferation of astrocytes characterized by swollen processes and watery cytoplasm (Ludwin, 1978). Ethidium bromide causes slow demyelination and is highly variable in the amount of demyelination induced (Graca and Blakemore, 1986; Yajima and Suzuki, 1979). Diphtheria toxin-induced demyelination is accompanied by extensive scarring and Wallerian-type degeneration of nerve fibers (Eames et al., 1977).

Experimental allergic encephalomyelitis (EAE), a possible experimental model for multiple sclerosis (MS), is also useful in inducing primary demyelination. However it is difficult to control focally, triggers an immune response in which the pathology varies, causes the proliferation of astrocytes, and causes some axonal degeneration (Raine and Bornstein, 1970a; Raine and Bornstein, 1970b).

Phototargeted ablation of oligodendrocytes has been quite successful in causing focal demyelination of the spinal dorsal columns of mice (Vanderluit et al., 2000). Unfortunately, this technique is limited to a transgenic mouse model expressing  $\beta$ -galactosidase from a cell specific promoter, and could not be used for clinical studies of regeneration.

There are several genetic mutant mouse models, such as jimpy, rumpshaker, trembler, and zitter, that show abnormal myelin development and significant hypomyelination (for review see Lunn et al., 1995). However, these mutants are marred

by complications such as unstable oligodendrocyte populations, abnormal oligodendrocytes and astrocytes, excessive glial cell death, and poor viability of animals (< 2 weeks). Due to these various complications, these models are unsuitable for studies of CNS injury and repair.

In order to study the effects of myelin removal on axonal regeneration, an effective technique that does not have complications like those described in the models above should be used. The technique of utilizing antibody and guinea pig complement in the study of experimental demyelination has evolved substantially in recent years. Characterization of galactocerebroside has demonstrated that GalC stains specifically for oligodendroglia, and that GalC is located abundantly within the oligodendrocyte lipid membrane (Benjamins et al., 1987). Upon application of antibody to inhibit GalC function, dramatic structural changes are induced in the oligodendroglia membrane, such as contraction of membrane sheets and the reorganization of the underlying cytoskeleton (Dyer and Benjamins, 1988). Furthermore, application of this antibody has also been shown to cause an influx of calcium in oligodendrocytes, leading to the suggestion that this influx may be mediating the cytoskeletal changes (Dyer and Benjamins, 1990). These observations were the basis for the hypothesis that GalC could be a good target for antibody-mediated attack of myelin and oligodendrocytes in attempts to induce demyelination.

*In vitro* studies have shown that anti-GalC antibody application inhibits myelin formation very specifically in a complement-dependent manner in rat spinal cord cultures (Bornstein and Raine, 1970), and in neonatal rat cerebellum cultures (Dorfman et al., 1979). Furthermore, Ranscht et al (1982) have used a monoclonal antibody against GalC

to prevent the appearance of peripheral myelin sheaths in cultures of rat sensory neurons and Schwann cells. These findings confirmed the belief that anti-GalC antibody application could be effective for myelin suppression.

Several *in vivo* studies involving direct application of anti-GalC serum to CNS myelin have also resulted in demyelination. Sergott et al (1984) showed that anti-GalC antiserum causes rapidly progressive, complement-dependent focal demyelination when injected into the guinea pig optic nerve, resulting from primary damage to oligodendrocytes. Carroll et al (1984) used microinjection of GalC antiserum into the optic nerve to induce primary demyelination by 2 days, followed by oligodendrocyte remyelination by 10 days. Mastaglia et al (1989) used micro-injection of anti-GalC serum into the spinal cord dorsal columns of rats to induce primary demyelination with a marked depletion of oligodendrocytes. These findings further supported the theory that anti-GalC antibody and complement application could induce substantial demyelination by demonstrating that these reagents are effective *in vivo*.

Based on these findings, previous work in the Steeves laboratory has focused on the development and utilization of a transient, immunological, myelin-specific, GalC antibody-mediated, complement-dependent technique that selectively targets myelin for destruction in an attempt to remove some of the axonal growth inhibition activity associated with CNS myelin (Dyer et al., 1998; Keirstead et al., 1995; Keirstead et al., 1992; Keirstead et al., 1998a; Keirstead et al., 1997). As mentioned, the onset of myelination in the developing chick spinal cord usually begins around embryonic day 13 of the 21 day developmental period. The embryonic chick has a permissive period of repair prior to E13, which is coincident to the beginning of the myelination of fibre tracts.

A single injection of serum complement proteins and anti-GalC antibodies into the thoracic spinal cord of an E9-E12 chick embryo resulted in a delay in the onset of spinal cord myelination until E17, which extended the normal permissive period for axonal regeneration and functional recovery to later stages of development (Keirstead et al., 1992; Keirstead et al., 1997). Thoracic cord transections as late as E15 resulted in complete neuroanatomical repair and functional recovery in these animals. In the mature spinal cord of posthatchling chicks, the same immunological protocol transiently demyelinates and disrupts compact myelin, thereby facilitating some axonal regeneration of axotomized avian brainstem-spinal neurons, albeit at a reduced level of repair when compared to the injured developing spinal cord (Keirstead et al., 1995). The compacted form of myelin subsequently re-appears after the immunological protocol is discontinued (Keirstead et al., 1995; Steeves et al., 1994). More recent work in the adult rat spinal cord indicates that a similar immunological treatment after a thoracic cord lesion also facilitates anatomical regrowth of axonal projections from specific brainstem-spinal nuclei (Dyer et al., 1998) as well as ascending dorsal column neurons (Keirstead et al., 1998a). These studies indicate that this GalC antibody and complement-mediated immunological protocol is effective in transiently removing CNS myelin from both the developing and adult spinal cord of different species, and can facilitate some functional axonal regeneration in these cases.

### **1.3) The Complement Cascade**

#### *1.3a) Introduction*

The complement system is a major effector pathway of the humoral immune

system. It is involved in the control of inflammation, the activation of phagocytes, and the lytic attack on cell membranes (reviewed in Law and Reid, 1989). The ability of complement to trigger a powerful, multifaceted response against foreign microbial invasion is critical for host defense against infection. Complement consists of a complex collection of approximately 17 plasma soluble proteins, many of which are zymogens (i.e. inactive pro-enzymes that require proteolytic cleavage to become active). These molecules are primarily derived from hepatocytes (Alper et al., 1969), which secrete the proteins directly into the blood supply. As the CNS is isolated from the systemic circulation by the Blood-Brain-Barrier (BBB), and most of these complement proteins are too large to cross the intact BBB, there are also extrahepatic sites of complement biosynthesis, such as astrocytes and microglia. Astrocytes are able to synthesize the entire repertoire of complement proteins, whereas microglia can only produce a limited number (C1q, C4, C3) (Morgan and Gasque, 1997). Activation of complement can occur along two possible pathways, the classical and alternative pathways, both of which result in the formation of the membrane attack complex (MAC).

### *1.3b) The Classical Pathway*

Activation of the classical complement pathway is initiated by complement-fixing antibodies (Figure 1). (A brief overview of classical pathway activation follows. For a more detailed review, see Devine, 1991; Tomlinson, 1993). Typically, the binding of two IgG antibodies to specific cell surface antigens (e.g. GalC), initiates the classical pathway. The first component of the classical pathway is C1, a calcium-dependent complex of the three proteins C1q, C1r, and C1s. Each C1 molecule consists of two

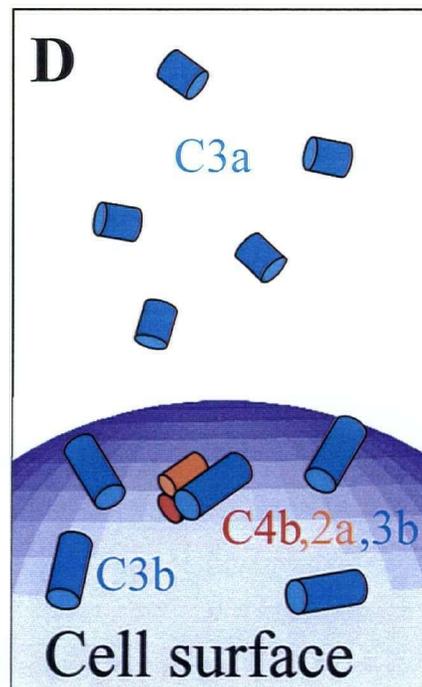
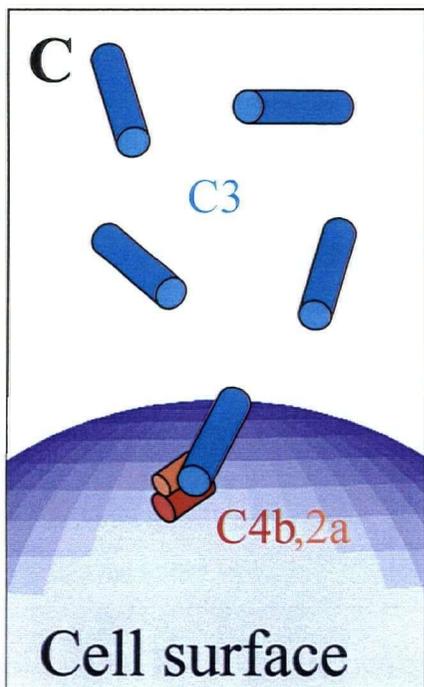
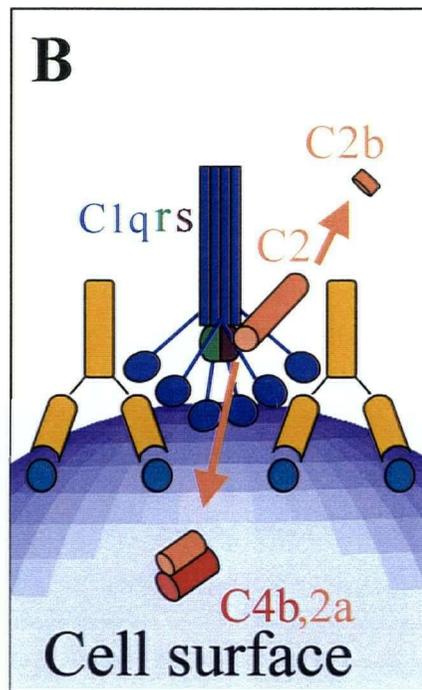
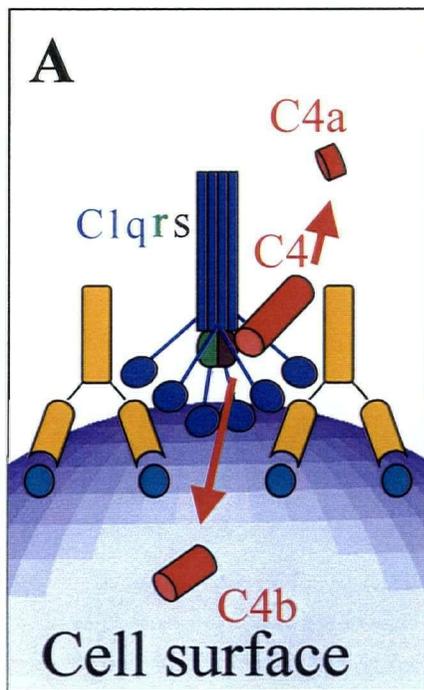
subunits each of C1r and C1s, and six C1q subunits. The C1q molecule has six globular heads joined to a common stem. Each of these heads can bind to one Fc region of an antibody, and the binding of two or more of these heads at the same time will activate the C1q molecule. C1q can be bound by either IgM or IgG antibodies. However, in the case of IgG, the binding energy required for activation can only be achieved when two IgG molecules are bound within 40 nm of each other (Burton, 1990). Because of this structural requirement for binding, these antibodies are not capable of activating complement in solution. The binding of C1q then activates the associated C1r, which becomes an active enzyme that cleaves the proenzyme C1s, generating a serine protease that initiates the classical complement cascade.

The activated C1s protease then acts on the next two components of the pathway, C4 and C2. First, the C4 molecule is cleaved to generate a small C4a fragment, which acts as a weak anaphylatoxin (a molecule that triggers a local inflammatory response), and a larger C4b fragment (Figure 1A), which binds covalently to the surface of the pathogen through a highly active thioester bond that is exposed upon cleavage (Chan et al., 1984). This allows the C4b molecule to bind to molecules in the immediate vicinity of its site of activation, such as a foreign pathogen. If C4b is not bound quickly, the exposed thioester bond will be cleaved by hydrolysis and C4b will be irreversibly inactivated. This process allows complement activation to occur on foreign pathogens but prevents it from affecting host-cell surfaces.

Once anchored to the membrane, C4b binds C2, which is then cleaved by C1s (Figure 1B) to produce larger fragment C2a, a serine protease which remains bound to C4b on the pathogen surface, and smaller fragment C2b, a precursor for a vasoactive

**Figure 1. The classical pathway of complement activation generates a C3 convertase that deposits large numbers of C3b molecules on the target surface.** The steps in the reaction are outlined here and explained in more detail in the text. (A) Activated C1s cleaves C4 to C4a and C4b, which binds to the target surface. (B) C4b then binds C2, which is cleaved by C1s, to C2a and C2b, forming the C3 convertase C4b,2a. (C) C4b,2a cleaves C3 to C3a and C3b, which binds to the target surface or to the convertase itself, forming the C5 convertase C4b,2a,3b. This convertase initiates the formation of the lytic MAC, which inserts into the target membrane, causing cell lysis and death. (D) One C4b,2a molecule can cleave many molecules of C3 to C3b, which then bind to the target surface. C3b opsonization is important for phagocytic ingestion of the target by macrophages. (Adapted from Janeway Jr. and Travers, 1997).

# Classical Pathway



molecule. C2 is susceptible to cleavage by C1s only when bound by C4b, thereby confining it to the pathogen surface. The C4b2a complex, the central enzyme of the cascade, remains on the surface as the C3 convertase of the classical pathway, cleaving C3 to C3a, which is an anaphylatoxin, and to C3b (Figure 1C), which either remains bound to C4b2a to form a C5 convertase or is deposited on the cell membrane through a reactive thioester bond that is exposed upon cleavage (Figure 1D).

C3 is the most abundant of the complement proteins in plasma (Erdei et al., 1991). The absence of C3 and the components of the classical complement pathway C3 convertase seriously impairs immune homeostasis and may lead to immune disease. This has been shown in cases of genetic deficiencies for complement factors C1, C2, C3, and C4 described in humans, guinea-pigs, and dogs (Burger et al., 1986; Rother, 1988), and in animals made C3 deficient by the use of cobra venom factor (Pepys, 1974). Effects of complement cascade activation include the deposition of large amounts of C3b on the pathogen surface in order to facilitate the destruction of the pathogen by macrophages. (The coating of a foreign particle with antibody or complement for ingestion by phagocytes is called opsonization.) In addition, C3b also activates the alternative pathway of the complement cascade. (These two phenomena will be discussed in more detail below). A third important function of C3b is that it binds to C4b of the C3 convertase, forming a 3-protein C5 convertase, C4b2a3b.

This C5 convertase, C4b2a3b, binds C5 by 3b and makes it susceptible to cleavage by the serine protease activity of C2a. The products of this cleavage are C5b, which goes on to begin the formation of the lytic membrane attack complex (MAC), and C5a, an anaphylatoxin (Figure 3A). This is followed by a series of reactions that leads to

the insertion of the MAC, which ultimately causes cell lysis and death via pore formation, as described below.

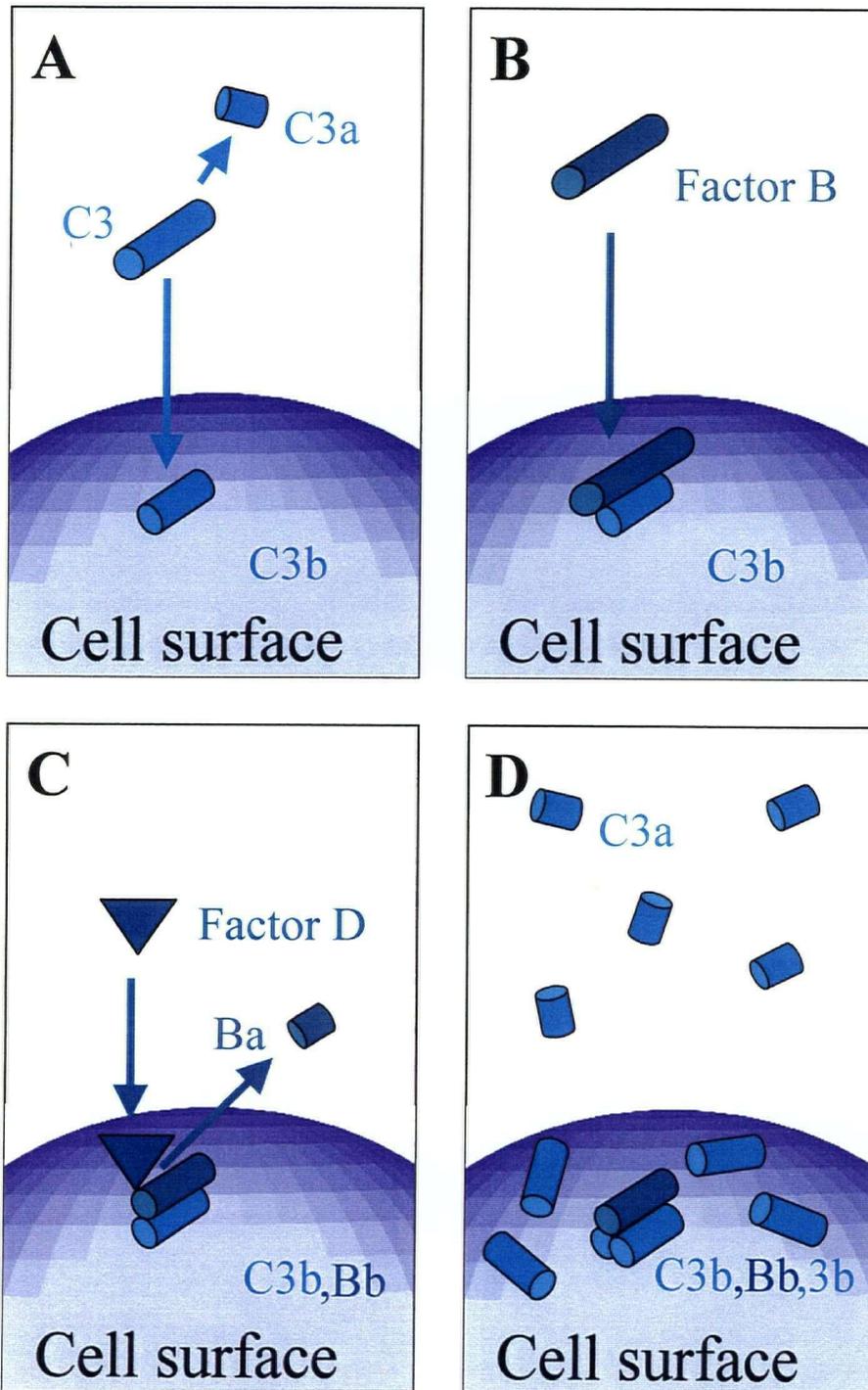
In the immunological, complement-dependent, anti-GalC antibody-mediated demyelination protocol, the GalC antibody would bind to antigen GalC on the myelin surface, thereby directing the classical pathway of complement activation to target the myelin membrane. C1 would then bind the Fc region of the antibody, with subsequent activation of the rest of the complement cascade. C3b may be deposited on the myelin surface in amounts sufficient to promote the stripping and phagocytosis of the myelin by invading or resident phagocytic cells.

### *1.3c) The Alternative Pathway*

The alternative pathway uses similar C3 and C5 convertase formation and activation in its action. (A brief overview of alternative pathway activation follows. For a more detailed review, see Devine, 1991; Tomlinson, 1993). The initiation molecule for the alternative pathway is C3b, which has multiple mechanisms of activation. First, it can be made by the cleavage of C3 that occurs in the classical pathway, in which case the subsequent activation and completion of the alternative pathway complements the effects of the classical pathway. Second, C3b can be activated in response to cell membrane-foreign antigen contact, with the spontaneous cleavage of the C3 component of complement to generate the active C3b molecule. This molecule then becomes surface bound, where it can initiate the completion of the alternative pathway. Third, there can also be a continuous hydrolysis of C3 in the extracellular fluid, resulting in soluble C3b. In these latter two scenarios, the alternative pathway becomes activated in an antibody-

**Figure 2. The alternative pathway of complement activation can amplify the classical pathway by depositing more C3b, or act on its own by spontaneous cleavage of C3 in the absence of specific antibody.** The steps in the reaction are outlined here and explained in more detail in the text. (A) C3 is cleaved spontaneously or by the classical pathway and C3b is deposited on the target surface. (B) C3b binds Factor B. (C) Bound Factor B is cleaved by Factor D into Ba and Bb, forming the C3 convertase C3b,Bb. (D) One C3b,Bb molecule can cleave many molecules of C3 to C3b, which then bind to the target surface, or to the convertase itself to form the C5 convertase C3b,Bb,3b. This convertase initiates the formation of the lytic MAC, which inserts into the target membrane, causing cell lysis and death. C3b opsonization is important for phagocytic ingestion of the target by macrophages. (Adapted from Janeway Jr. and Travers, 1997).

# Alternative Pathway



independent manner. In all cases, the alternative pathway begins with the deposition of C3b on the membrane surface (Figure 2A). Membrane-bound C3b then binds Factor B in a magnesium-dependent manner (Figure 2B), and Factor B is in turn cleaved by the plasma protease Factor D to produce a C3 convertase C3bBb, and releases a small fragment, Ba (Figure 2C). As in the classical pathway, the C3 convertase of the alternative pathway can cleave many molecules of C3 to generate even more active C3b on the pathogen surface (Figure 2D). It should be noted that C3b is structurally and functionally homologous to C4b, the first fragment to bind to the pathogen surface in the classical pathway, and factor B is homologous to C2. Binding of another C3b to C3bBb forms the alternative C5 convertase, C3bBb3b. This C5 convertase can cleave C5 into C5b, which initiates the generation of the membrane-attack complex (MAC), and C5a, a potent mediator of inflammation (Figure 3A). This is followed by a series of reactions that leads to the insertion of the MAC, which ultimately causes cell lysis and death via pore formation, as described below.

In the immunological, complement-dependent, anti-GalC IgG antibody-mediated demyelination protocol, C3b may be deposited on the myelin membranes through any of the aforementioned scenarios, such as classical pathway activation, spontaneous cleavage in response to a foreign cell type, or continuous spontaneous hydrolysis. The subsequent activation of the alternative pathway through to MAC insertion would follow. Since the activation of the alternative pathway can be independent of antibody, C3b deposition could be occurring on other, untargeted cell types such as astrocytes, microglia, or neurons. Thus, it would be advantageous to exclude the alternative pathway from the infusion cocktail of this protocol in order to prevent any uncontrolled, nonspecific

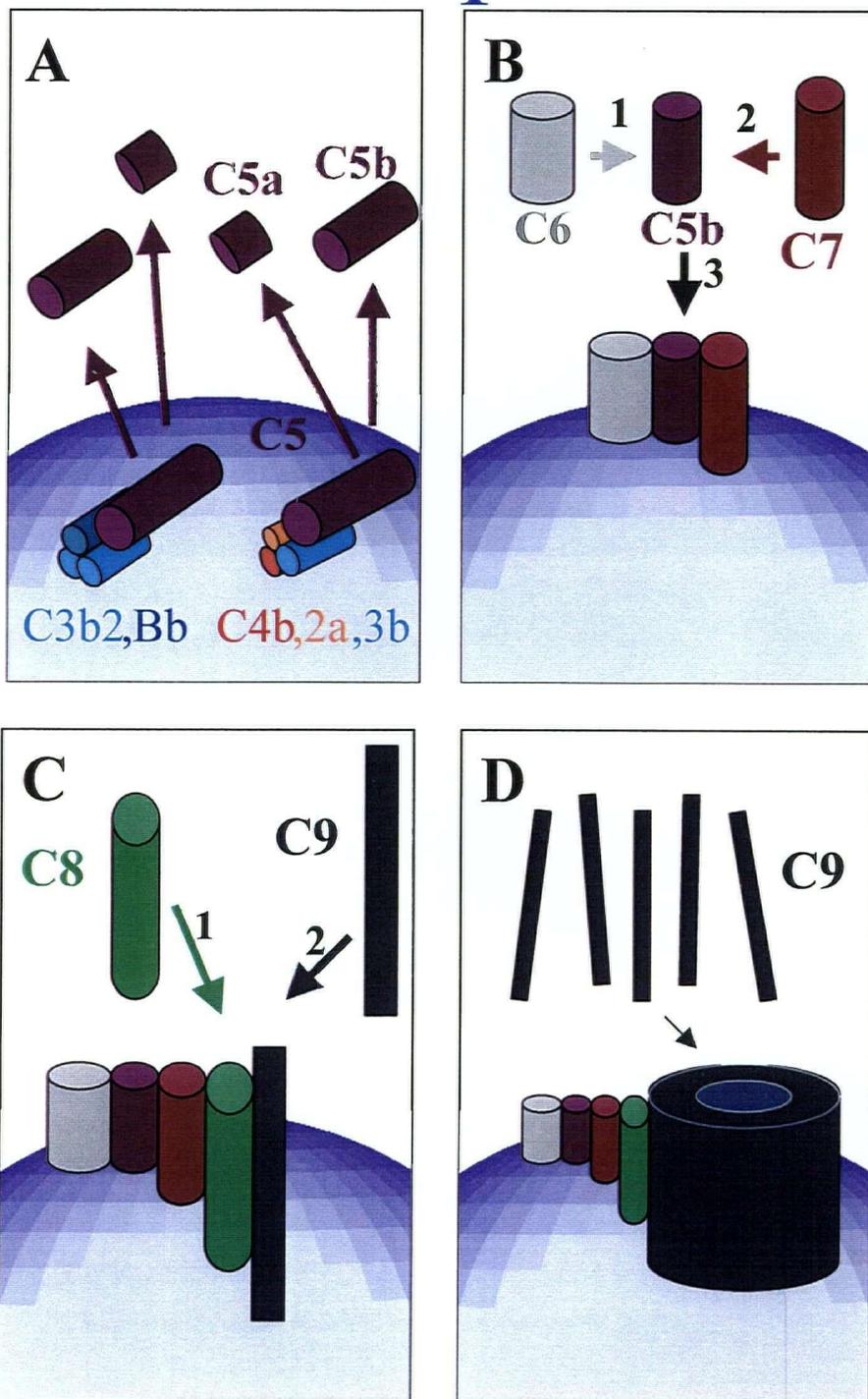
damage that may be occurring upon its delivery to the spinal cord.

### *1.3d) The Membrane-Attack Complex*

Both the classical and alternative pathways have a common ending, the lytic MAC pathway (Figure 3). (A brief overview of MAC activation follows. For a more detailed review, see Bhakdi and Trandum-Jensen, 1991; Shin and Carney, 1988). Unlike the classical and alternative branches of the cascade, MAC formation is nonenzymatic, but relies on the hydrophilic-amphiphilic transitions of the terminal complement proteins. The common initial MAC component is the soluble C5b, which can be formed by the C5 convertases C4b2a3b and C3bBb3b of the classical and alternative pathways, respectively (Figure 3A). One molecule of C5b then binds one molecule of C6, inducing the expression of a binding site for C7 (Figure 3B). This reaction leads to a conformational change in the constituent molecules, with the exposure of a hydrophobic site on C7. The hydrophobic domain of C7 inserts into the lipid bilayer (Figure 3B), resulting in exposure of a binding site for C8 on C5b. Subsequently, one molecule of C8 binds to the membrane associated C5b67 complex at C5b, and inserts into the membrane through a subsequent exposure of a hydrophobic site similar to that described for C7 insertion (Figure 3C). Finally, C8 induces the polymerization of 10 to 16 extracellular C9 molecules to complete pore formation and membrane penetration (Figures 3C and 3D), allowing free passage of solute and water across the lipid bilayer through its hydrophilic internal channel. This results in a loss of cellular homeostasis, and gives potentially toxic extracellular molecules (e.g. Ca<sup>2+</sup>) access to the cytoplasm of the target membrane (e.g. myelin). Insertion of the MAC in sufficient amounts leads to membrane lysis and death

**Figure 3. Complement component C5 is activated by C5 convertases of the classical and alternative pathways, and then proceeds to allow for the assembly of the membrane attack complex to generate a pore in the lipid bilayer membrane.** The steps in the reaction are outlined here and explained in more detail in the text. (A) C5 binds to C3b of the C5 convertases C4b,2a,3b and C3b,Bb,3b of the classical and alternative pathways, respectively, forming C5b and C5a. (B) C5b binds C6, which allows it bind C7, and the C5b,6,7 complex attaches to the target membrane via C7. (C) C8 binds to the complex and inserts into the target membrane, allowing C9 to do the same. (D) Several molecules of C9 bind to form a pore in the membrane, allowing free passage of solutes across membrane. This leads to cell lysis and death of the targeted cell. (Adapted from Janeway Jr. and Travers, 1997).

# Membrane Attack Complex



of the targeted cell.

In the immunological, complement-dependent, anti-GalC antibody-mediated demyelination protocol, the MAC may be inserted into the target myelin membrane via either the classical or alternative pathways of complement activation, resulting in rupture of the myelin membrane, and subsequent demyelination of the surrounded axon.

### *1.3e) Inflammation and Anaphylaxis*

The anaphylatoxins C3a, C4a, and C5a are cleavage products of the complement cascade that are known to act as proinflammatory mediators (for reviews of anaphylatoxins, see Frank and Fries, 1991; Gerard and Gerard, 1994; Tomlinson, 1993). C4a is similar to C3a in structure but has a lower affinity for its receptors, making it the weakest anaphylatoxin of the three. C3a induces smooth muscle contraction, enhances vascular permeability, causes the release of vasoactive substances such as histamine through its actions on basophils, and is chemotactic for many cells, including phagocytes (Hartmann et al., 1997; Schaber et al., 1994; Tomlinson, 1993). Receptors for C3a are involved in initiating its inflammatory effects, and are found on a variety of cells, including macrophages, monocytes, mast cells, eosinophils, neutrophils, and basophils (Erdei et al., 1991).

C5a by far has the strongest activity of the three anaphylatoxins, being shown to be 200-fold stronger than C3a and 3000-fold stronger than C4a in causing inflammatory reactions through histamine release and smooth muscle responses (Hugli et al., 1981). Perhaps more importantly, C5a is strongly chemotactic for many cells including mononuclear phagocytes, basophils, eosinophils, and neutrophils (Cui et al., 1994; Nolte

et al., 1996), increases neutrophil adhesiveness and aggregation (Schulman et al., 1988; Swerlick et al., 1988), and is known to increase phagocytic ability and increase the expression of complement receptors CR1 and CR3 on macrophage surfaces (Gerard and Gerard, 1994). C5a has high affinity for its phagocytic cell surface receptors, and as such, C5a's effects are apparent at relatively low concentrations (Huey and Hugli, 1985). Furthermore, the inflammatory response of astrocytes and microglia may be mediated by chemotaxis and cell activation by C5a (Armstrong et al., 1990; Yao et al., 1990).

In the immunological, complement-dependent, anti-GalC antibody-mediated demyelination protocol, anaphylatoxins C3a, C4a, and C5a, may be acting to promote local inflammation at the infusion site, thereby attracting phagocytic cells to the region and facilitating the removal of myelin.

#### **1.4) Complement and the Nervous System**

Recently, complement has been implicated in various pathologies of the nervous system. Complement components are thought to play a role in demyelinating diseases of both the CNS and PNS, such as MS and Guillain-Barré syndrome (GBS), respectively. Multiple sclerosis is characterized by progressive loss of the myelin sheath from axons within the CNS, with consequent neural conduction deficits. It is now widely believed that MS is an autoimmune disease of the nervous system driven by infiltrating T cells specific for antigens on CNS myelin, with myelin loss being attributed to the activity of infiltrating macrophages (Prineas and Wright, 1978). Soluble complement activation complexes have been detected in the cerebrospinal fluid (CSF) of MS patients, and MAC deposits were found in the brains of MS patients, particularly associated with the edges of

active plaques (Compston et al., 1989; Mollnes et al., 1987; Yam et al., 1980). These findings suggest that complement may be contributing to the development of the symptoms observed in MS patients.

Evidence for complement activation in MS has also emerged from studies using the animal model of MS, EAE. In EAE animals, de complementation with cobra venom factor markedly inhibited disease symptoms (Linnington et al., 1989). In an antibody-dependent form of EAE, de complementation with cobra venom factor or inhibition of complement with soluble complement receptor 1 inhibited demyelination and helped prevent disease (Piddlesden et al., 1994). These studies indicate that complement may be a key factor in creating MS-like symptoms in these animals.

In the PNS, clinical neuropathologies such as GBS and Miller-Fisher syndrome are characterized by extensive demyelination and inflammation. GBS patients have myelin antibodies which can activate complement (Koski, 1990; Koski et al., 1986), and complement activation products including C3a, C5a, and the MAC have been found in the CSF, plasma, and peripheral nerves of patients (Hartung et al., 1987; Hays et al., 1988; Koski et al., 1987). This suggests that the complement cascade may contribute to PNS demyelinating pathologies as well.

Some neurodegenerative diseases in which neuronal loss without demyelination or inflammation is the primary characteristic also involve complement activation. Alzheimer's disease (AD) is characterized by senile plaques and neurofibrillary tangles caused by the accumulation of abnormal protein fibrils (Hardy and Allsop, 1991). These protein accumulations stained strongly for components of the classical complement system (Eikelenboom et al., 1989; Eikelenboom and Stam, 1982; McGeer et al., 1989).

The nature of activation is unclear, and may involve only early complement proteins (C1, C4, C3) (Veerhuis et al., 1995) or the complete cascade including the MAC (McGeer and McGeer, 1992). Similar results were found for Pick's disease, another degenerative disease characterized by the buildup of dense amorphous bodies (Singhrao et al., 1996; Yasuhara et al., 1994). These findings suggest that the complement cascade may be involved in other neurodegenerative diseases that normally are not characterized by demyelination.

Thus, further elucidation of the role of complement and its various components in demyelination may be valuable for understanding the underlying mechanisms of pathologies involving complement-mediated demyelination or neural degeneration.

### **1.5) Complement In Host Defense and Disease: The Independent Activity of Complement Pathways**

There are several examples in host defense and disease states involving complement activation that suggest the classical and alternative pathways can act independently of one another, or that only one pathway is sufficient for effective action, and that these pathways can sometimes act without MAC insertion and lysis.

In many cases, the classical pathway of complement activation acts independently of the alternative pathway. In Guamanian parkinsonian dementia and in AD, diseases characterized similarly by the appearance of neurofibrillary tangles in cortical and subcortical areas (Braak et al., 1999), extracellular tangles were intensely stained with antibodies for classical pathway proteins C1, C4, and C3, but not alternative pathway proteins (Schwab et al., 1996). Studies of complement-dependent phagocytic attack of

*Legionella pneumophila* by human polymorphonucleocytes (PMN) and macrophages using antibody and sera deficient in C2, C3, or C5 showed that neutralization occurred via opsonization, which was dependent on antibody-mediated activation of the classical pathway, with no detection of alternative pathway or MAC activation (Verbrugh et al., 1985). *Borrelia burgdorferi* is resistant to the bactericidal action of complement in the absence of specific antibody or C2, suggesting that cell killing is occurring through the classical pathway (Kochi et al., 1993). These findings suggest that the alternative pathway is not always required or sufficient for the complement cascade to exert its effects.

The alternative pathway has also been shown to activate independently of the classical pathway. In defense against the *enterococci* bacterium, the alternative pathway of complement activation was sufficient for neutrophil-mediated killing of bacteria, since antibody was not required and C4-deficient serum exhibited similar bactericidal activity to normal human serum (Harvey et al., 1992). In studies on defense against malaria-causing *Plasmodium gallinaceum*, lysis was mediated by the alternative pathway, independent of classical pathway activation (Touray et al., 1994). Studies of the lysis of horse red blood cells by chicken complement demonstrated independence from antibody for lysis, dependence on magnesium but not calcium for lysis to occur, lack of consumption of C1 when lysis was observed, and the inability of a C1 inactivator to prevent lysis, all indicating that lysis is caused by alternative pathway activation independent of the classical pathway (Ohta et al., 1984). Host defense killing of *Tritrichomonas foetus* by antibody and complement was abolished by depletion of factor B by heat treatment, demonstrating the necessity of the alternative pathway to this

process (Aydintug et al., 1990). These studies indicate that the classical pathway is not always necessary or sufficient for complement-mediated cell killing to occur.

In some cases, either the classical or alternative pathway or both can act without the insertion of the lytic MAC. Studies of *Hemophilus influenza*, which is susceptible to complement lysis, indicate that host defense is far more dependent on the opsonization properties of C3 than on the lytic capacity of the complement cascade (Noel et al., 1988). In rabbits with anti-glomerular basement membrane glomerulonephritis, MAC proteins are not necessary for the full expression of injury in the presence of immune cells, even though complement activation contributes to the injury (Tipping et al., 1989). In studies examining the effects of complement on antibody-mediated EAE demyelination, depletion of C3 and C5 had no effect on the clinical severity of the disease, suggesting that the demyelination observed is acting independent of the MAC, and the major role of the complement cascade in this case appears to be the generation of pro-inflammatory factors that enhance the inflammatory response and cause an antibody-dependent cell-mediated cytotoxic response (Piddlesden et al., 1991). Normal human serum is highly cytotoxic for amastigotes of *Leishmania major*, which causes Oriental sore (Kubba et al., 1987). This cytotoxicity is mediated by the alternative complement pathway, but was shown to be independent of MAC formation in experiments using human sera genetically deficient in C5, C6, C7, C8, or C9 (Hoover et al., 1985). These findings suggest that the complement cascade can sometimes act independently from the MAC.

The above examples demonstrate that the entire complement cascade is not necessary for all complement-mediated processes to occur effectively.

### **1.6) Hypothesis and Experimental Plan**

Based on this information, I hypothesized that the entire complement cascade is not required for complement-mediated, GalC antibody-dependent demyelination within the adult rat spinal cord, and that removing certain components such as the alternative pathway proteins would still result in substantial demyelination. To test this hypothesis, I have investigated the contributions of the classical and alternative pathways in the complement cascade, and also of the particular complement proteins themselves (C3, C4, Factor B, and C5), to the focal CNS demyelination observed in response to the immunological demyelination protocol developed in Dr. John Steeves' laboratory (Keirstead et al., 1992). By infusing serum specifically deficient for each of these proteins together with anti-GalC antibody, I have rendered either the classical, alternative, or MAC pathways inactive. Through examination of axonal-myelin ultrastructure, I was then able to determine whether or not the complete complement cascade directly caused the observed myelin changes. Furthermore, I was able to assess the roles of the classical, alternative, or MAC pathways of complement activation in demyelination. This work may add a valuable demyelination protocol to clinical and research science, and could help elucidate the role of complement in demyelinating neuropathologies and immune-mediated phenomena.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

## 2.1) Choice of Depleted Sera

Human complement sera were obtained from Sigma Inc (St. Louis, MO). Deficient sera were prepared and verified by Sigma Inc using affinity chromatography, a highly sensitive hemolytic assay, and Ouchterlony microimmunodiffusion (Kolb et al., 1979). All full or deficient human complement sera were infused into the intact spinal cord along with a complement-fixing IgG antibody to GalC (see below). Sera deficient for a specific protein were selected on the basis of their potential interruption of specific pathways within the complement cascade (Figure 4). C4-deficient serum (Sigma # C-0913) (n=4 animals) was used to test the role of the classical pathway, Factor B-deficient serum (Sigma # C-0535) (n=4) to test the role of the alternative pathway, C3-deficient serum (Sigma # C-8788) (n=4) to test the importance of the convergent point of the two pathways in the complement cascade with the membrane attack complex, as well as the possible role of macrophage chemotaxis, and finally, C5-deficient serum (Sigma # C-1163) (n=2), to examine the necessity of the MAC to demyelination. Normal (i.e. intact) human serum (Sigma # C-9473) (n=4) was used as a positive control. Other control animals received an infusion of the polyclonal IgG anti-GalC alone (n=4), or received no treatment (n=2).

## 2.2) Antibody Production

Polyclonal rabbit IgG antibody to GalC was provided by Dr. Claire Huguenot of CORD at UBC according to a protocol described by Ranscht et al (1982). Briefly, adult New Zealand rabbits (6) were immunized subcutaneously with 500  $\mu$ L Incomplete Freund's Adjuvant, 300 $\mu$ g of Keyhole Limpet Haemocyanin (KLH), and 150  $\mu$ g of GalC

I and II, in a total volume of 1 mL. Booster injections were given twice at 3 week intervals. After assessment of an antibody response, a further booster injection, without KLH, was given 3 months after the initial injection. One month later a total bleed was performed. After clotting, protein was precipitated with ammonium sulphate, and dialysis performed against binding buffer for Protein A/G purification. Samples were then purified to the IgG fraction on a Protein A/G column (Calbiochem IP10) according to the manufacturer's instructions. Samples were purified for anti-GalC IgG fractions on an affinity column conjugated with GalC. After elution, samples were diluted to 0.5 mg/mL for storage.

### **2.3) Transient Immunological Myelin Disruption**

Adult male rats (Sprague-Dawley, 10-12 weeks old), approximately 220 g in weight, were anaesthetized with intraperitoneal injections of Ketamine/Xylazine (60 mg/kg, 7.5 mg/kg respectively). A dorsal incision was performed and lidocaine was injected into the musculature surrounding the dorsal aspect of the spinal column. After a limited dorsolateral laminectomy, an intraspinal cannula was implanted at T10 (n=22 total) and connected to an Alzet osmotic mini-pump (Model 2002; Alza Corp, Palo Alto, CA.) to subsequently deliver a continuous intraspinal infusion (@ 0.5  $\mu$ l/hr, for 7d) of one of the various complement sera (33% v/v, see above) along with a complement-fixing IgG antibody to galactocerebroside (0.25 mg/mL), diluted in sterile 20 mM PBS. Each cannula was held in place by means of dental acrylic applied to the vertebral bone and to a stainless-steel screw inserted at T11. The osmotic pump was then placed in a subcutaneous cavity. Muscle layers were then sutured over the dental acrylic, and the

superficial tissue and skin closed. Surgical procedures were performed by Dr. Jason Dyer of CORD at UBC. All surgical procedures and subsequent animal care protocols were in accordance with Canadian and UBC Animal Care Committee guidelines.

#### **2.4) Electron Microscopy**

Tissue for ultrastructural analysis was obtained from 10-12 week old adult male Sprague-Dawley rats sacrificed at 7-days post-implantation of the mini-osmotic pumps. Animals were lethally anaesthetized with Ketamine/Xylazine (120 mg/kg, 15 mg/kg respectively), and then perfused intracardially with 200 mL of 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 100 mL of 4% glutaraldehyde in 0.1 M PB (pH 7.3), and subsequently post-fixed overnight in the same fixative. The infusion site and surrounding spinal cord was cut into 1mm transverse blocks and processed to preserve rostral-caudal sequence. Blocks were washed in 0.1 M sodium cacodylate buffer (24 hours), post fixed in 2% OsO<sub>4</sub>, dehydrated through increasing concentrations of ethanol (70%, 85%, 95%, 100%, 100%) and propylene oxide, and embedded in Spurr's resin according to standard protocols. Tissue blocks from experimental and untreated-control animals were processed in parallel. Semithin sections (1 µm) were cut from each block, mounted on Superfrost slides, stained with 2% alkaline Toluidine Blue and examined under a light microscope. Semithin sections were examined for evidence of demyelination/inflammation in order to determine the regions of tissue to be processed for ultrastructural examination. For electron microscopic examination, blocks were trimmed and ultrathin sections (approximately 25 sections per 1 mm transverse block) cut

at 80-100 nm, mounted on copper grids, stained with 2% uranyl acetate and lead citrate and viewed under a Zeiss EM 10C electron microscope (at 80 kV).

**Figure 4. Cartoon of complement cascade that may be involved in immunological demyelination, indicating experimental complement serum-deficiency points.** Two pathways of activity are possible in the complement cascade: the Classical Pathway (molecules C1, C4, and C2 at bottom of diagram), which is antibody mediated, and the Alternative Pathway (molecules B and D at top of diagram), which is mediated by the spontaneous conversion of C3 to C3b. Both pathways converge to form the Membrane Attack Complex (MAC). This results in the permeabilization of the cell membrane and cell lysis.

Complement proteins (C4, Factor B, C3, C5) removed from the human serum complement used in these experiments are highlighted with a yellow circle at their respective positions in the cascade .

Infusing serum deficient in C4 (red) with GalC antibody should halt the classical pathway of the complement cascade after the binding of C1 to the GalC antibody, thereby rendering this pathway inactive. The alternative pathway (including opsonizing deposition fragment C3b), and the MAC (including anaphylatoxin C5a), should still have the ability to activate through to completion.

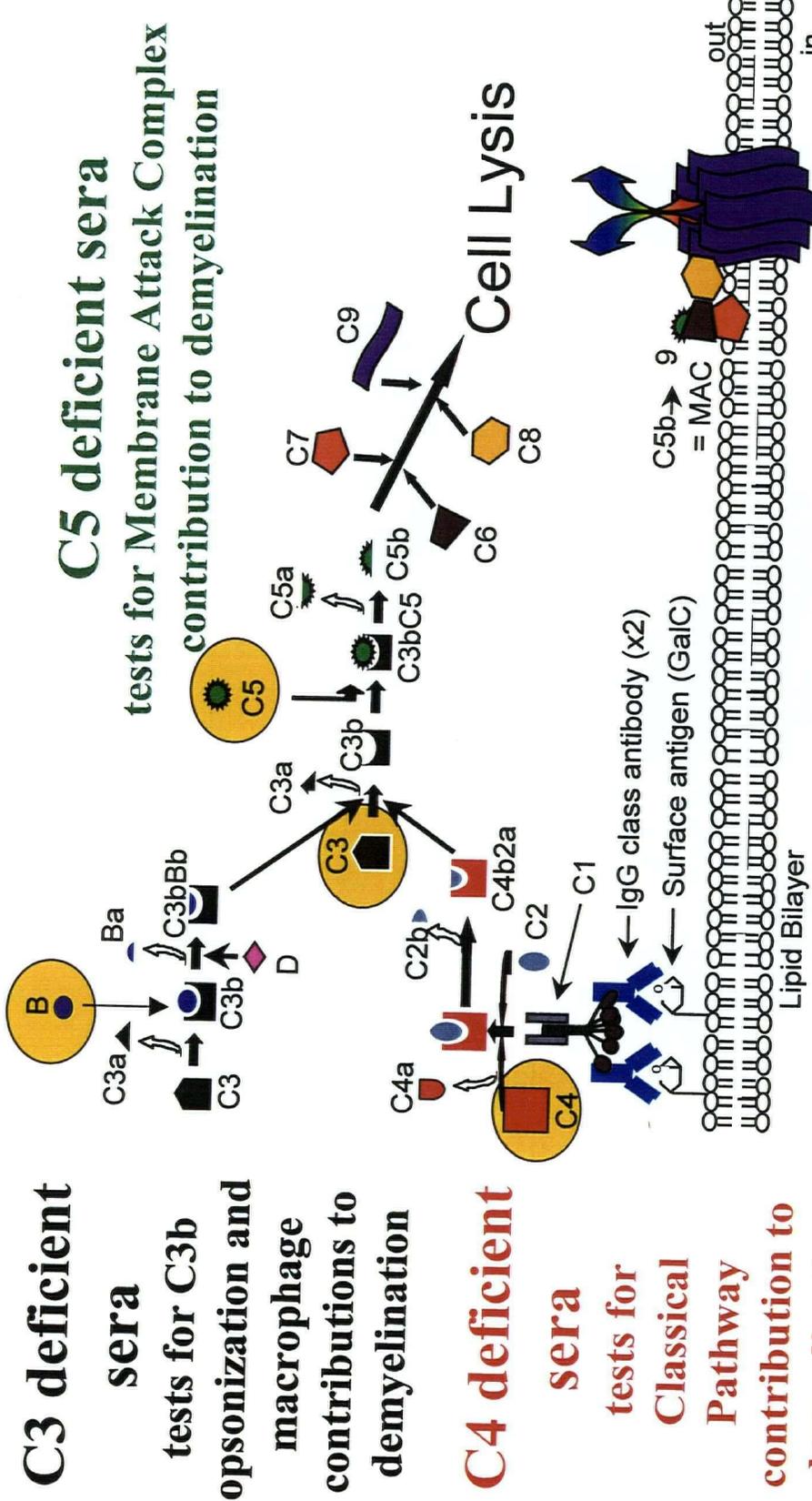
Infusing serum deficient in Factor B (blue) with GalC antibody should halt the alternative pathway of the complement cascade after initial C3 deposition, thereby

rendering this pathway inactive. The classical pathway (including opsonizing deposition fragment C3b and anaphylatoxins C4a and C3a), and the MAC (including anaphylatoxin C5a), should still have the ability to activate through to completion.

Infusing serum deficient in C3 (black) with GalC antibody should halt the classical pathway of the complement cascade after C4b2a deposition and the alternative pathway before initiation, thereby rendering both pathways inactive. Only C4b2a and anaphylatoxin C4a should be active, and no C3 opsonization should take place.

Infusing serum deficient in C5 (green) with GalC antibody should halt the complement cascade after deposition of the C5 convertases (C4b2a3b and C3b,Bb,3b) from both the classical and alternative pathways, thereby preventing MAC assembly. The classical pathway (including opsonizing deposition fragment C3b and anaphylatoxins C4a and C3a), and the alternative pathway (including C3b), should still have the ability to activate through to completion. However, the MAC (including anaphylatoxin C5a) should be unable to form.

**Factor B deficient sera**  
 tests for Alternative Pathway  
 contribution to demyelination



**C3 deficient**

**sera**  
 tests for C3b  
 opsonization and  
 macrophage  
 contributions to  
 demyelination

**C4 deficient**

**sera**  
 tests for  
 Classical  
 Pathway  
 contribution to  
 demyelination

**C5 deficient sera**

tests for Membrane Attack Complex  
 contribution to demyelination

**CHAPTER 3**

**RESULTS**

Results are based on the ultrastructural analysis of the adult rat spinal cord following the direct intraspinal infusion of myelin-specific IgG antibody along with human serum complement, either whole complete serum (as a positive control) or serum depleted through immunoadsorption for a specific complement enzyme (Figure 4). Since the implantation of an osmotic pump cannula can cause some focal nonspecific damage at the site of its dorsal lateral cord insertion, all ultrastructural assessments of spinal myelin integrity were made from the adjacent dorsal column region, which was quite distinct from the cannula insertion site. Immunological demyelination does not lend itself easily to quantification, since reliable quantification normally requires counts of myelinated and demyelinated axons, measurements of lesion areas, assessment of remyelination, and analyses of axonal damage, spinal cord atrophy, astrocytic response, and inflammatory foci. This is beyond the scope of this study, and therefore, all observations remain descriptive.

Direct intraspinal (T10) infusion over 7 days (0.5  $\mu$ l/h) with normal 33% human serum complement along with an IgG polyclonal antibody to GalC (0.25 mg/mL) in PBS resulted in profound demyelination within the dorsal column of all animals, extending to 1 mm on either side of the cannula insertion site (Figure 5B). Axons appeared relatively normal, and some mature oligodendrocytes were observed to survive within this demyelination zone. Oligodendrocytes were identified on the basis of their association with many axons, oval nuclei with typically clumped heterochromatin around the nuclear envelope, and relatively densely-stained, thin cytoplasm, which distinguishes them from astrocytes, which are more lucent under electron microscopy (Peters et al., 1976). In addition, large numbers of macrophages (or activated microglia) containing myelin debris

were present within the demyelinated spinal tissue. Macrophages were identified on the basis of their irregular nuclei with heterochromatin typically clumped around the nuclear envelope, high-density endoplasmic reticulum, finger-like processes, and phagosomes containing engulfed myelin debris or lipid breakdown products. These macrophages/microglia contained myelin ovoids and were closely associated with the naked axons. In addition, a few blood-borne cells such as neutrophils or other polymorphonucleocytes were observed to infiltrate the infusion region (not shown). At rostral-caudal distances further removed from the infusion site ( $> 2$  mm), myelin disruption, as characterized by a delaminated appearance, was observed.

Control infusions of human serum complement alone (Figure 5D) or antibodies to GalC alone (Figure 5C) showed normal myelination comparable to that of untreated animals (Figure 5A). Minimal myelin disruption was observed at the infusion site, and the compact nature of the myelin was preserved.

Upon direct intraspinal infusion of human serum complement deficient in C4, an enzyme specific for the classical complement pathway, along with the IgG anti-GalC in PBS, normal myelination was observed at and around the infusion site (Figure 6A), and a normal distribution of spinal neural cells and processes was found throughout all cords examined. Myelin appeared similar to that observed after control infusions of human serum complement alone or IgG anti-GalC antibody alone (as shown in Figures 5D and 5C).

Infusion of human serum complement deficient in Factor B, an enzyme specific for the alternative complement pathway, along with the IgG anti-GalC in PBS resulted in a demyelinated CNS environment in all animals tested (Figure 6B), similar to that observed

after infusion of normal human complement along with the IgG anti-GalC (as shown in Figure 5B). Myelination was suppressed up to 1 mm on either side of the infusion site, and large regions of naked axons were observed, once again associated with activated macrophages/microglia (Figure 7B). These axons maintained a normal appearance at the ultrastructural level, characterized by maintained integrity of axonal microtubules and cellular organelles. Demyelinated axons were observed 1 mm to either side of the infusion site, and myelin disruption a further 2-3 mm rostral and caudal from the cannula insertion point.

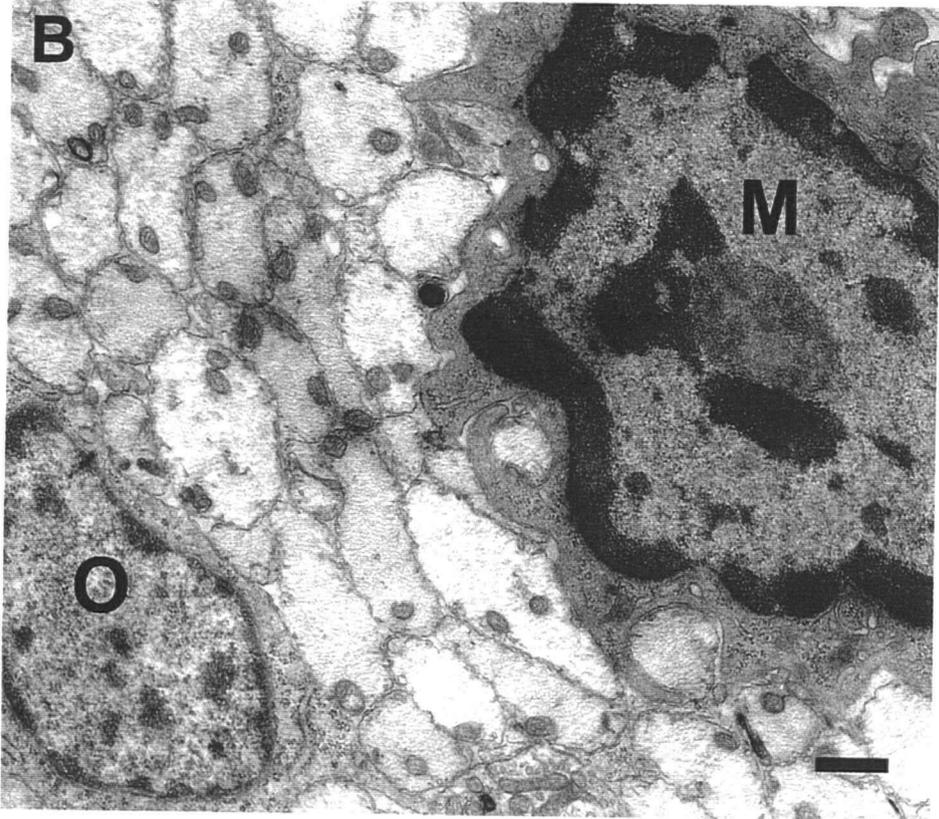
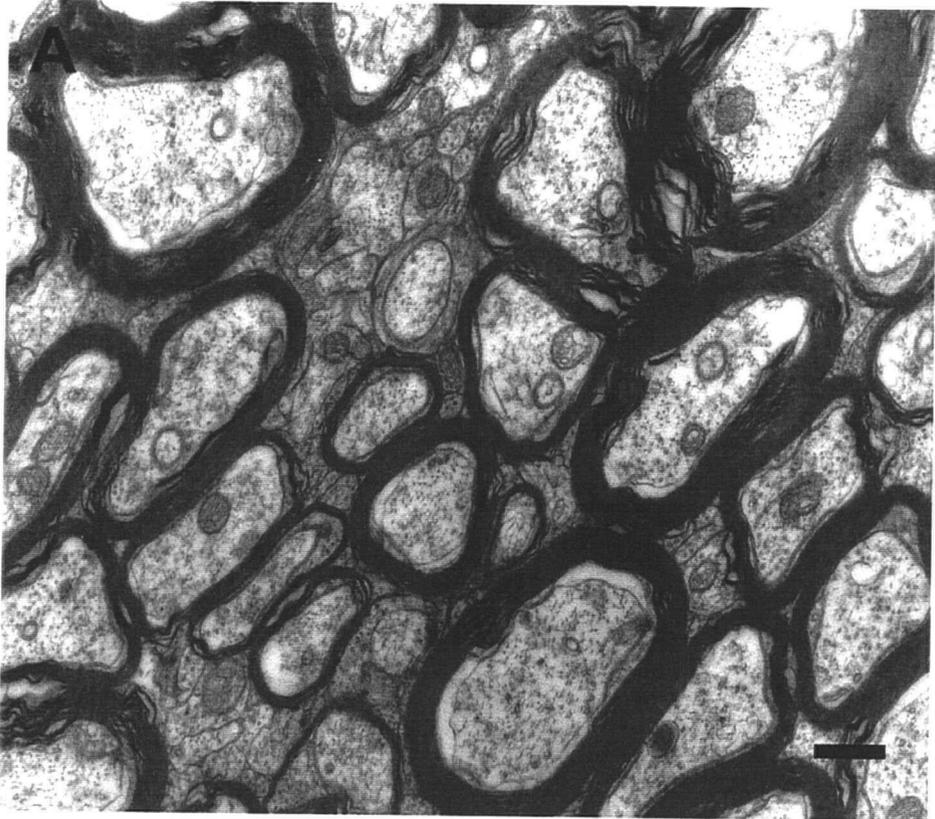
Infusion of the IgG anti-GalC and human complement deficient in C3, a protein common to both complement pathways, resulted in a distinct lack of any type of immunological reactivity in the dorsal column of all animals tested (Figure 7A). Axons and oligodendrocytes appeared healthy, as did all the neural cell types. Spinal myelin retained its compact nature with little, if any, disruption, similar to that observed after control infusions of human serum complement alone or IgG anti-GalC alone (as shown in Figures 5D and 5C).

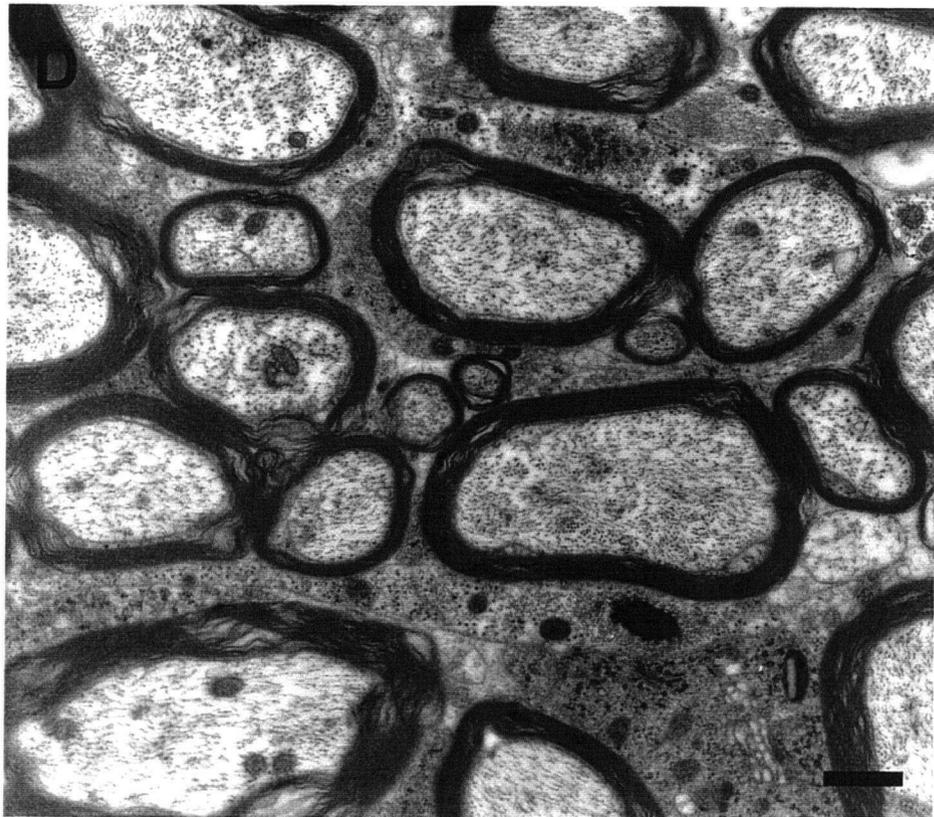
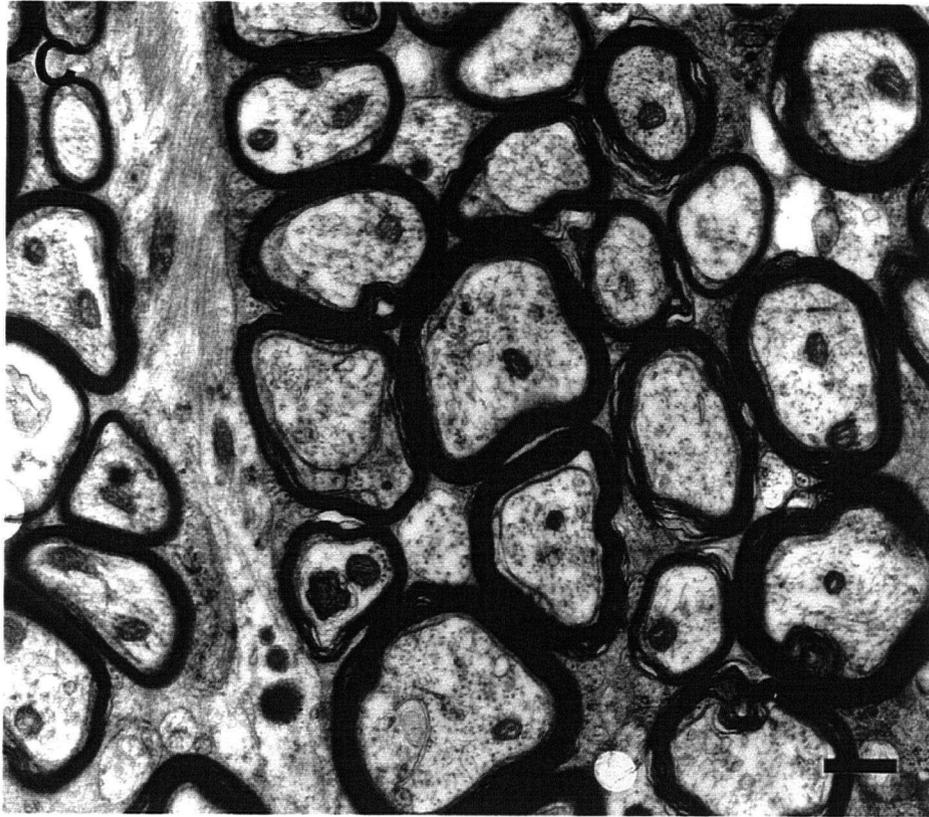
Direct intraspinal infusion of IgG anti-GalC and human complement deficient in C5, an early membrane attack complex protein, resulted in substantial demyelination within the dorsal columns up to 1 mm on either side of the infusion site in all animals tested (Figure 8), similar to that observed after infusion of normal human complement along with the IgG anti-GalC (as shown in Figure 5B). This zone of induced demyelination was accompanied by the presence of macrophages containing myelin debris. All axons appeared normal at the ultrastructural level.

Demyelinated lesions observed upon individual infusion of the various complement sera (e.g. complete human complement, Factor B-deficient complement, C5-deficient complement) together with GalC antibody were fairly consistent across all animals tested. The demyelinated region had a rostral-caudal distance of 1.5-2.0 mm in all cases. At further distances of 2-3 mm, delamination of myelin was observed. At the light microscope level, effects were observed throughout the entire dorsolateral quadrant of the infusion site, sometimes extending into the more ventral regions of the cord. Macrophages were much more prevalent at the infusion site, but were also found associated with naked axons at more distal demyelinated regions. Overall, the demyelinated axons themselves maintained their integrity, defined by round profiles, with normal mitochondria and intact neurofilaments and microtubules, and minimal degenerated axon profiles were observed. However, moderate neurofilament disarray and microtubule disassembly accompanied by decreases of axonal calibre, all common traits of demyelinated lesions (Blakemore, 1984; Blakemore, 1978; Blakemore, 1975; Blakemore, 1977; Blakemore et al., 1977; Dal Canto and Lipton, 1980), were observed in some populations of demyelinated axons. These were usually found at or near the infusion site, where inflammatory reactivity and any trauma induced by cannula insertion would be greatest. Although these features call into question the health of these axons (Zhu et al., 1999) and their ability to function properly, they may also be indicative of a down-regulated axonal phenotype more conducive to growth (Keirstead et al., 1998a). In past studies, demyelinated axons have been shown to persist and support both regeneration (Dyer et al., 1998; Keirstead et al., 1995; Keirstead et al., 1998a; Keirstead et al., 1999; Keirstead et al., 1997) and remyelination (Blakemore, 1984; Blakemore,

1978; Blakemore, 1975; Blakemore, 1977; Blakemore et al., 1977; Dal Canto and Lipton, 1980), which could revert the axons to their original, normal appearance and function.

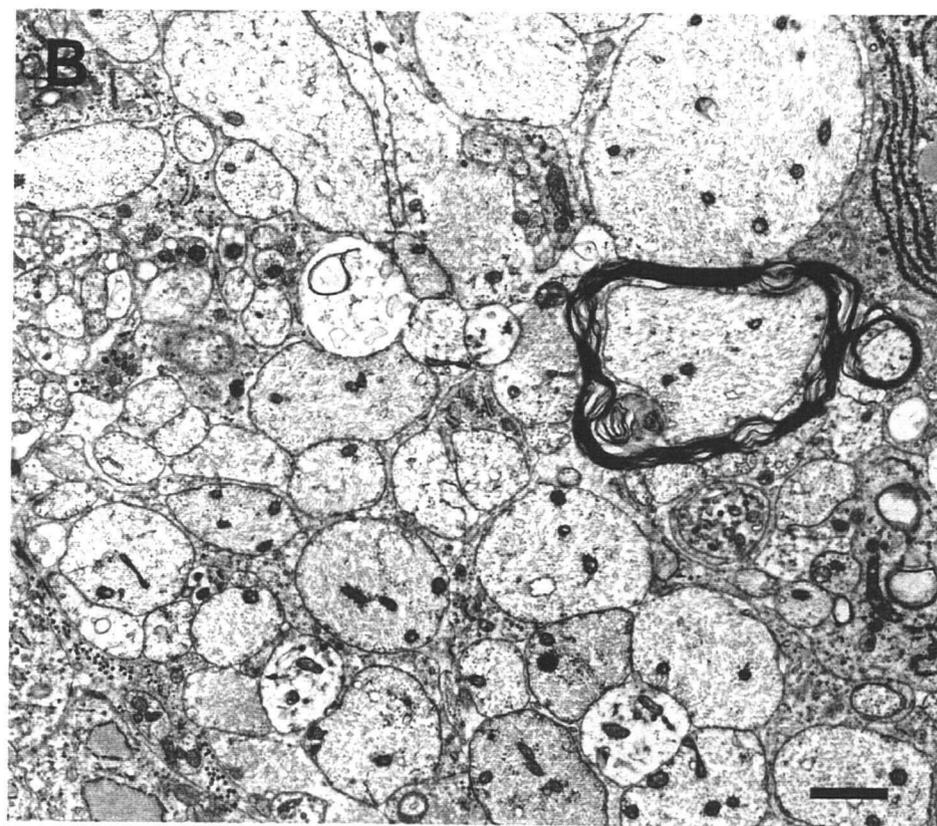
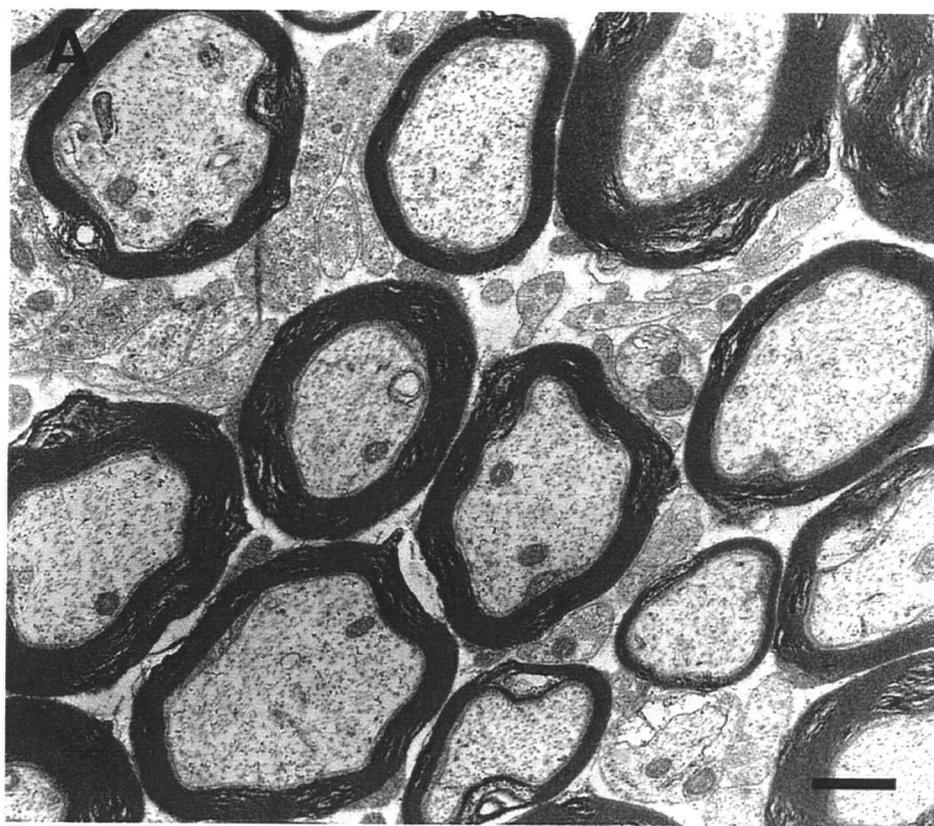
**Figure 5. The effects of complete human complement and anti-GalC IgG antibody infusion on myelin ultrastructure compared to control infusions of antibody alone or complement alone.** Electron photomicrographs of transverse sections through dorsal columns of adult rat thoracic spinal cord. (A) Control untreated animals showing normal appearance of myelin. (B) After 7 days of continuous intraspinal infusion of full human complement serum and anti-galactocerebroside antibody, large regions of demyelinated axons were visible within 1 mm of the infusion site. Oligodendrocytes (O), appearing normal, are observed. Macrophages (M) are observed among regions of healthy naked axons. (C, D) Myelin structure following 7-day infusion of galactocerebroside IgG antibody alone (C) or human complement alone (D) within 1 mm of infusion site, showing compact nature of myelin with minimal nonspecific damage. Scale bars = 1  $\mu$ m.



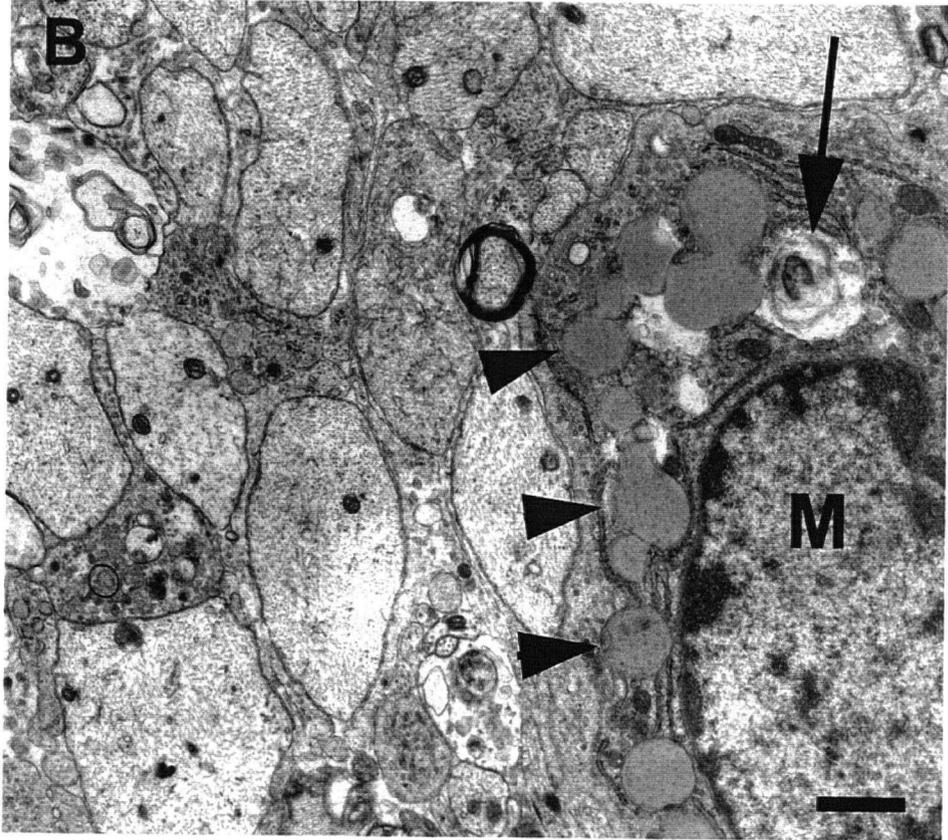
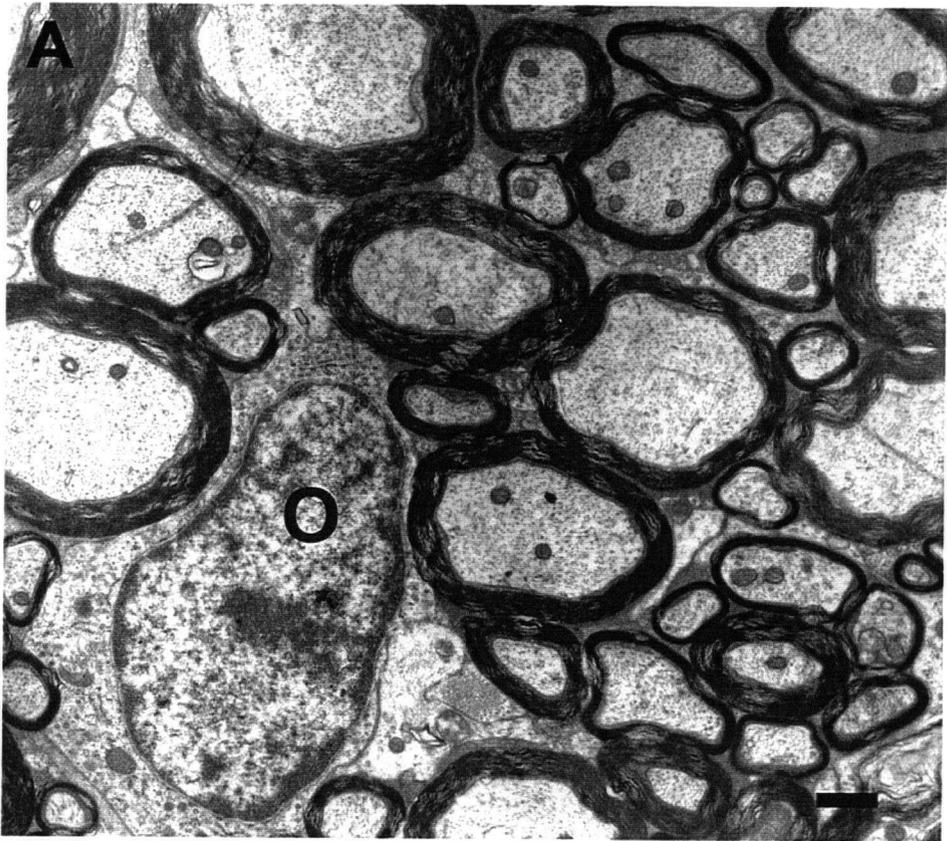


**Figure 6. Classical and alternative pathway contributions to demyelination. (A)**

Electron photomicrograph of a transverse section through the dorsal column of adult rat thoracic spinal cord following 7-day infusion of galactocerebroside antibody with human complement deficient in C4, within 1 mm of infusion site. The compact nature of myelin remains, with minimal disruption and no demyelination observed, similar to that seen for control animals. (B) Electron photomicrograph of a transverse section through the dorsal column of adult rat thoracic spinal cord following 7-day infusion of galactocerebroside antibody with human complement deficient in Factor B, within 1 mm of infusion site. Extensive demyelination is observed, with axons appearing otherwise healthy, similar to that seen for treatment with complete human complement and anti-GalC IgG antibody. Occasional axons are seen with disrupted thin myelin sheaths. Scale bars = 1  $\mu$ m.

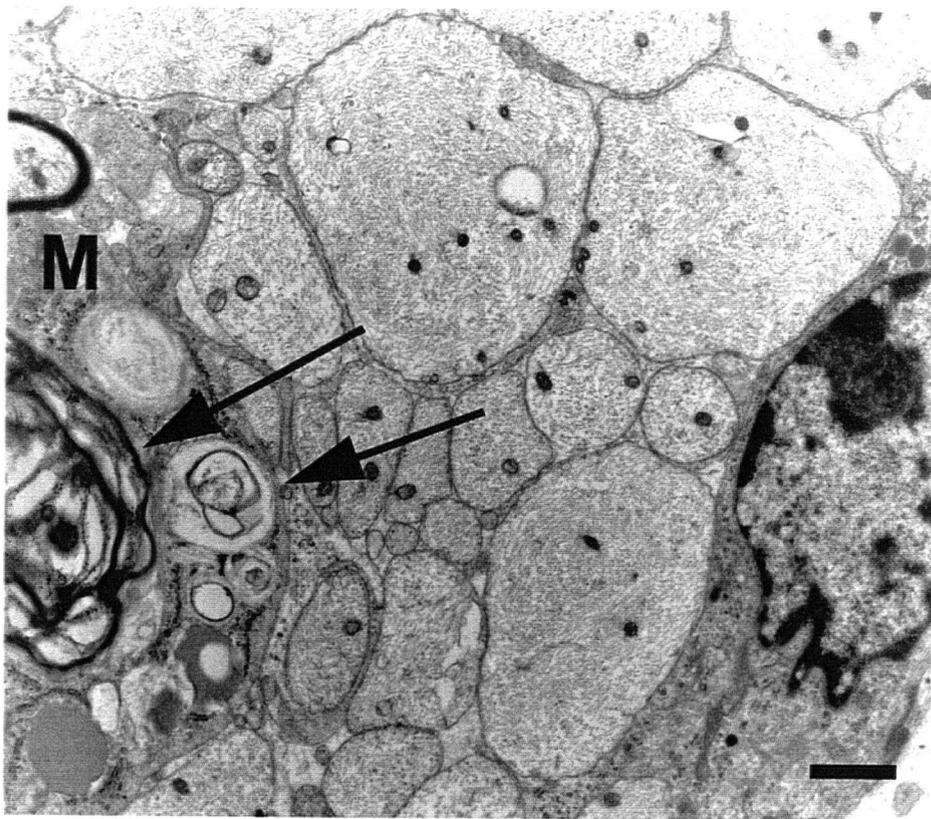


**Figure 7. C3 and macrophage contributions to demyelination.** (A) Electron photomicrograph of a transverse section through the dorsal column of adult rat thoracic spinal cord following 7-day infusion of galactocerebroside antibody with human complement deficient in C3, within 1 mm of infusion site. Normal myelin is observed, with minimal disruption and no demyelination, similar to that seen for control animals. Oligodendrocytes (O) and axons again appear healthy, and there is no evidence of macrophage activity. (B) Electron photomicrograph of a transverse section through the dorsal column of adult rat thoracic spinal cord following 7-day infusion of galactocerebroside antibody with human complement deficient in Factor B, within 1 mm of infusion site, as in Figure 6B. Naked demyelinated axons are associated with macrophages (M), which contain lysosomes containing myelin breakdown products (arrowheads) or myelin debris (arrow). Scale bars = 1  $\mu$ m.



**Figure 8. The effects of C5 deficient serum and anti-GalC IgG infusion on myelin.**

Electron photomicrograph of a transverse section through the dorsal column of adult rat thoracic spinal cord following 7-day infusion of galactocerebroside antibody with human complement deficient in C5, within 1 mm of infusion site. Extensive demyelination is observed, with axons appearing otherwise healthy, similar to that seen for treatment with full human complement and GalC antibody. Again, naked demyelinated axons are associated with macrophages (M), which contain myelin ovoids or myelin debris (arrows). Scale bars = 1  $\mu$ m.



**CHAPTER 4**

**DISCUSSION**

The presence of CNS myelin has been shown to be inhibitory to axonal regeneration, and some myelin-associated inhibitory molecules, such as myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994) and Nogo (or NI35/250) (Caroni and Schwab, 1988c), have been identified. Because of this, a technique that causes a transient removal of these proteins, or myelin itself, in a specific manner could prove to be valuable in the facilitation of axonal regeneration.

Complement and myelin-specific antibody have been used to induce demyelination in the past. Antibody-directed complement fixation has been shown to alter the ionic balance of many different cells *in vitro* (Mayer, 1972). Previous *in vitro* experiments using inactivated peritoneal macrophages showed that myelin phagocytosis occurred readily in the presence of specific anti-GalC antibodies, but not when complement alone was added (Scolding and Compston, 1991). When serum complement and myelin-specific antibodies were applied to purified oligodendrocyte cultures, myelin suppression was observed (Dubois-Dalcq et al., 1970). *In vivo*, anti-GalC mediated, serum complement-dependent myelin disruption in the guinea pig optic nerve occurred within 1-2 hours of treatment (Sergott et al., 1984). These studies suggest that GalC-mediated complement attack of the myelin sheath may be an effective way of removing myelin.

Previous work from Dr. John Steeves' laboratory has used a transient, immunological, myelin-specific, GalC antibody-mediated, complement-dependent protocol to suppress or remove myelin in order to promote the regrowth of severed CNS axons. This protocol was successful in suppressing the onset of myelination of the developing avian spinal cord (Keirstead et al., 1992; Keirstead et al., 1997), demyelinating/disrupting myelin of the mature avian cord (Keirstead et al., 1995), and

demyelinating the myelin of the adult rat cord (Dyer et al., 1998; Keirstead et al., 1998a), thereby facilitating significant anatomical regeneration of injured axons with accompanying functional recovery in these animals (Keirstead et al., 1995). Further examination of the mechanism of demyelination in these cases is necessary to determine which of the infused immunological reagents are essential for demyelination.

In the present study, I have examined the mechanisms of the immunological demyelination induced by the protocol developed in the Steeves laboratory in greater detail by analyzing the specific proteins and pathways involved in the complement cascade. It might prove beneficial to remove unnecessary proteins from the infusion protocol to prevent unwanted side effects caused by these proteins from occurring, and to allow for the development and refinement of this technique for clinical purposes. I have observed that the entire complement cascade is not required for significant demyelination to occur within the adult rat spinal cord.

In previous studies, guinea pig complement and an IgG anti-GalC antibody were utilized to induce transient myelin suppression/demyelination (Dyer et al., 1998; Keirstead et al., 1995; Keirstead et al., 1992; Keirstead et al., 1998b; Keirstead et al., 1997). Control infusions (anti-GalC antibody alone, complement alone, vehicle alone) did not cause any demyelination of the spinal cord in these cases, indicating that both serum complement and a myelin-specific, complement-fixing antibody are essential for this immunological CNS demyelination to occur. In consideration of the time course of demyelination, it was observed that disruption of the myelin sheath occurs within one day of the onset of intraspinal infusion of guinea pig complement and IgG anti-GalC (unpublished observations). After 3d post infusion, macrophages were observed within

the infused spinal tissue, and within 7d post infusion, significant demyelination of a large region of axons was observed. This demyelination extended up to 2 mm on either side of the insertion site for the infusion cannula, beyond which myelin disruption (delamination) was observed. The definite beginnings of remyelination were apparent within 14d. These findings suggested that 7d would be an appropriate time point for studies of the complement proteins that may be contributing to this demyelination, since considerable demyelination is evident by this time, remyelination has not yet begun, and macrophage infiltration and myelin clearance is well under way.

*In vivo* studies have shown that many oligodendrocytes survive the anti-GalC-mediated, serum complement-dependent demyelination caused by the utilization of this protocol (Keirstead et al., 1998b). This may be because GalC is found in greater concentration on the surface of myelin membranes than on oligodendrocyte cell bodies (Ranscht et al., 1982). The abundance of GalC on the myelin membrane makes it an ideal target for this immunological protocol, since the binding of the two IgG molecules necessary to initiate the complement cascade requires a high concentration of antigen (Law and Reid, 1989). Recent evidence suggests that after the immunological infusion is terminated, remyelination begins within two weeks (Keirstead et al., 1998b), and the new myelin originates primarily from differentiating oligodendrocyte progenitors or precursors, although invading Schwann cells and surviving mature oligodendrocytes may also contribute to the observed remyelination.

In the study presented here, I have utilized human complement, which is readily available as deficient sera. Similarly, human serum complement is more applicable to the development of a clinically relevant neurotrauma reagent than guinea pig complement.

The use of sera deficient for specific complement enzymes allowed me to dissect the roles of the classical pathway of complement activation, the alternative pathway, and the MAC in the demyelinating process. Seven-day intraspinal infusion of complete human serum complement along with a myelin-specific IgG anti-GalC antibody resulted in focal demyelination within 1 mm of the infusion site, with myelin disruption extended in both rostral and caudal directions to the infusion site. Regions of naked axons were found closely associated with macrophages or activated CNS microglia containing myelin debris. The precise origin of these phagocytic cells is unknown at present, but could be activated resident CNS microglia and/or invading systemic macrophages entering the CNS through a compromised blood-brain barrier resulting from the infusion of these vasoactive reagents. Preliminary work using dichloromethylene diphosphate, an agent that depletes systemic monocytes and thus inhibits blood-derived macrophages, suggests that macrophages are the primary mediators of myelin phagocytosis in the immunologically myelin-suppressed spinal cord, and though microglia may be contributing to the observed demyelination, they do not compensate for the absence of macrophages in removing myelin (Dyer et al., 1999). The demyelination observed in the present study was very similar to that described upon guinea pig complement and GalC antibody infusion (Dyer et al., 1998), with the exception that the demyelination is limited to within 1 mm of the infusion site, rather than the 2 mm range described previously. This could be advantageous in regeneration studies if a very localized region of demyelination is desired to minimize deleterious effects of unnecessarily demyelinated regions of the spinal cord.

Negative control infusions of complete (or normal) human complement alone or antibody alone had limited effect on myelin with no demyelination evident, indicating that both serum-complement and the GalC antibody are essential for this immunological CNS demyelination to occur. Furthermore, the lack of demyelination when the anti-GalC antibody alone was injected also suggests that the animal's endogenous serum complement is unable to compensate for the missing exogenously supplied sera.

Infusing serum deficient for C4, a protein positioned early in the classical pathway of the complement cascade, together with GalC antibody should halt the classical pathway of the complement cascade after binding of C1 to GalC antibody, thereby rendering this pathway inactive. The alternative pathway (including opsonizing deposition fragment C3b), and the MAC (including anaphylatoxin C5a), should still have the ability to activate through to completion. Infusion of GalC antibody and sera deficient for C4 resulted in normal myelination, (i.e., no demyelination) with minimal myelin disruption. Occasional aberrations in the myelin may be due to deposition of antibody and C1, which should still be occurring. The lack of demyelination indicates that C4 and, by extension, the classical pathway of complement activation, are essential to the demyelination protocol. The protein(s) in the pathway that is directly responsible for the demyelination remains to be determined, as C4 or any protein downstream from it could be responsible. Furthermore, these findings suggest that the alternative pathway of complement activation does not have the capacity to induce demyelination on its own in the absence of the classical pathway, and thus, is not a necessary component of the infusion. The lack of an immunological response when C4 is deleted from the composition also extends the observation that the animal's endogenous serum complement is unable to compensate for

an exogenously supplied sera that is deficient for the activation of the classical pathway. This lack of compensation may be at least partially due to cross-species incompatibility, since human and rat complement proteins in general, and human C2 and rat C4 specifically, have been reported to be incompatible (von Zabern, 1988). Both a compromised blood-brain barrier resulting from the experimental procedure, and activated macrophages and microglia (Morgan and Gasque, 1997), could be sources of endogenous complement in this situation. Although contributions from endogenous complement cannot be completely ruled out, these findings suggest that the infused complement is needed for at least the initiation of the demyelination process, since demyelination was not observed in control groups and some experimental groups (C3-deficient, C4-deficient).

Infusing serum deficient for C3 with GalC antibody should halt the classical pathway of the complement cascade after C4b2a deposition, and the alternative pathway before initiation, thereby rendering both pathways inactive. Only C4b2a and anaphylatoxin C4a should be activated, and no C3 opsonization will take place. Infusion of GalC antibody and sera deficient for C3 resulted in normal myelination, with minimal myelin disruption. Occasional aberrations in the myelin may be due to deposition of GalC antibody and the continuation of the classical pathway through to C4b2a deposition on the myelin membrane, which should still be occurring. Determining C3's importance to this demyelination protocol is critical due to its role in the pathway. This protein is common to both the classical and alternative complement pathways and becomes activated at the point where the pathways converge. C3 is the most abundant complement component in plasma, and the complement cascade becomes dramatically amplified through increasing

C3b deposition, which in turn will trigger further activation of the alternative pathway (Erdei et al., 1991). In addition to its role as the protein connecting the classical and alternative pathways to the MAC portion of the pathway, C3b is a major ligand for complement receptors on phagocytic cells, as are its inactive derivatives such as C3bi and C3dg (Erdei et al., 1991). The lack of demyelination (i.e., preservation of normal myelin) when C3-deficient sera and GalC antibody were used indicates that C3 is essential to the immunological demyelinating procedure, as would be expected if the complement cascade is required. Since the opsonization of the target membrane by C3b is necessary for the phagocytosis of myelin by microglia/macrophages (Erdei et al., 1991), these findings support the suggestion that microglia/macrophages are involved in the transient immunological demyelination procedure.

Infusing serum deficient for Factor B with GalC antibody should halt the alternative pathway of complement cascade after initial C3 deposition, thereby rendering this pathway inactive. However, the classical pathway (including opsonizing deposition fragment C3b and anaphylatoxins C4a and C3a), and the MAC (including anaphylatoxin C5a), should still have the ability to activate through to completion. Infusion of Factor B-deficient sera along with GalC antibody to restrict the contribution of the alternative pathway resulted in profound demyelination, similar to that observed in animals treated with complete human serum complement and GalC antibody. The observed demyelination was accompanied by the invasion of macrophages, which contained myelin ovoids and were closely associated with the naked axons. This suggests that the classical pathway of complement activation is sufficient for demyelination to occur. Combined with observations of C4 deficient experiments described above, these findings

also support the suggestion that Factor B and, by extension, the alternative pathway, have limited, if any, ability to trigger the observed demyelination in the absence of the classical pathway, and are therefore not necessary components of the infusion. This independence of classical pathway activation has been observed in other conditions. For example, the classical pathway is thought to act independently in disease states such as AD, in which there is distinct activation of the classical but not the alternative pathway (McGeer et al., 1989), although the cause of activation is unknown. In the present study, however, the alternative pathway may still be contributing to the demyelination observed upon whole human complement and IgG anti-GalC infusion by complementing the activity of the classical pathway. Reliable quantification of these results could provide insights into whether this is occurring by allowing comparisons of extent and/or rate of demyelination with and without the alternative pathway. Furthermore, immunolabelling of alternative pathway proteins to test for their association with myelin could also be done to examine alternative pathway contributions to demyelination in this case.

These findings are of particular significance with regards to this demyelination protocol because the alternative pathway is known to act as a first line of defense against pathogens in the absence of specific antibody (Janeway Jr. and Travers, 1997). In short, C3 is spontaneously cleaved by hydrolysis to produce C3b, which may bind covalently to surrounding cells, initiate the complement cascade, and cause nonspecific, untargeted damage to their environment. A study of nonspecific damage to cells in the infusion region was beyond the scope of the work presented here, and hence, untargeted damage was not directly observed. However, as the alternative pathway does not appear to be crucial for demyelination to occur upon administration of the reagents in this

immunological protocol, refinements of the composition, omitting alternative pathway proteins, would in future limit potential side effects caused by nonspecific damage that might result from activation of this pathway.

Infusing sera deficient for C5 with GalC antibody as the infusion cocktail should halt the complement cascade after deposition of the C5 convertases (C4b2a3b and C3bBb3b) from both pathways, thereby preventing MAC assembly. The classical pathway (including opsonizing deposition fragment C3b and anaphylatoxins C4a and C3a), and the alternative pathway (including C3b), should still have the ability to activate through to completion. However, the MAC (and anaphylatoxin C5a) should be unable to form. In order to determine if the demyelination observed previously is caused by MAC insertion and myelin membrane lysis, I used serum deficient for C5, an early MAC protein, and infused it intraspinally with GalC antibody. Extensive demyelination was observed, once again accompanied by macrophages containing myelin debris. These results suggest that the MAC is not necessary for the demyelination induced by this immunological protocol. Complement activity independent of MAC insertion has been observed in other demyelinating neuropathologies. For example, in rats with EAE, antibodies to myelin (MBP) penetrate into the CNS to produce demyelinated MS-like lesions in a complement-dependent manner (Linington et al., 1989; Pabst et al., 1971). However, the contributions of the MAC to the demyelination process are unclear. Antibody-mediated EAE demyelination has been shown to include membrane deposition of C9, a late MAC protein, on the target membrane (Linington et al., 1989). However, *in vivo* complement depletion studies suggest that the MAC is not essential to the pathogenesis of

demyelination in EAE (Piddlesden et al., 1991). These findings are similar to the results obtained in the present study.

Observations in these C5-deficiency experiments suggest that a mechanism other than membrane lysis via MAC insertion must contribute to the demyelination process. In some studies of complement cascade activity, the significance of the membrane-attack proteins and membrane lysis is debatable, and their contribution may in fact be quite limited. Therefore, one must consider the opsonization and inflammation activity of the earlier components of the cascade as potentially important contributors to any cellular activity involving the complement cascade. Such activity could explain the results obtained in the work presented here.

One possible explanation for the observed findings is that the myelin is directly stripped and engulfed by invading or resident phagocytic cells. Phagocytosis is a process that involves the recognition and binding of a target to the surface of a phagocytic cell, followed by the target's ingestion by the phagocyte. Complement's importance in the phagocytic process, and thus, in the clearance of microorganisms in host defense, has been clearly demonstrated in many *in vivo* animal studies and by the identification of individuals lacking the capability to cleave C3 (Brown et al., 1983). Furthermore, studies have indicated that host defense is generally more dependent on opsonization and phagocytosis than on the lytic capacity of the plasma (Noel et al., 1988). In all animals in which demyelination was observed in this study, naked axons were found associated with macrophages. Using the macrophage inhibitor dichloromethylene diphosphate with complete guinea-pig complement and anti-GalC IgG has been shown to prevent demyelination of the adult rat spinal cord (Dyer et al., 1999), suggesting that

macrophages are indeed essential for the demyelination observed in these experiments. Furthermore, in the present study, blood-borne cells such as neutrophils and PMNs, which act in inflammation and phagocytosis, were found to infiltrate the infusion area. Both PMNs and monocytes are present in the bloodstream, but are relatively ineffective at clearance there, and become much more effective at phagocytic ingestion once they enter the tissues (Frank and Fries, 1991). In the present study, phagocytes such as invading blood-borne macrophages, neutrophils, PMNs, and resident microglia could be responsible for the observed demyelination.

Microorganisms are opsonized by being coated with complement components that are recognized by specific receptors on phagocytic cells. The target then becomes immobilized at the phagocytic cell surface by this ligand-receptor interaction, thereby aiding the phagocytic process (Brown et al., 1983; Horwitz, 1982). Opsonization of pathogens is a major function of C3b and its proteolytic and inactive derivatives, C3bi and C3dg. C4b, the functional homolog of C3b, is also an opsonin, but its role is minor due to the relatively low amounts generated compared to C3b (Ross and Medof, 1985). In the immunological, complement-dependent, anti-GalC IgG antibody-mediated demyelination protocol described here, C3b may be deposited on the myelin surface through the activation of the classical pathway of the complement cascade, thereby preparing the myelin for targeted attack by infiltrating macrophages. This could be tested using complement receptor antibodies, C3b antibodies, or soluble C3b receptors to prevent demyelination-specific activation of the complement receptors on the surface of phagocytic cells.

Receptors for the C1q and the C3 degradation fragments C3b, C3bi, and C3dg have

been described on various cell types. The principal receptor for C3b (and C4b) is CR1. These receptors are prominent on phagocytic cells including macrophages and PMNs, as well as B cells (Erdei et al., 1991). It has been shown *in vitro* that macrophage phagocytosis of myelin is mediated by CR3 (Bruck and Friede, 1990). Other signals, such as the simultaneous presence of IgG molecules on a target surface that engage phagocyte Fc receptors, help initiate the phagocytic process via CR1. A further contribution to activation can be made by binding of the phagocyte to ECM-associated proteins such as fibronectin, laminin, and type I collagen (Griffin and Griffin, 1980; Newman and Tucci, 1990; Wright and Griffin, 1985). In addition, C3b is subject to regulatory mechanisms that cleave C3b into inactive derivatives such as C3bi and C3dg, which remain attached to the pathogen and act as opsonins for the binding of receptors CR2, CR3, and CR4 on phagocytic cell surfaces.

Most complement receptors of resting cells are mobile in the cell membrane. During the phagocytic process, the cells become active and the complement receptors become attached to the cytoskeleton, thereby losing their motility, and become capable of mediating ingestion (Janeway Jr. and Travers, 1997). The receptor CR1 is of this type and is normally part of an intracellular pool that cycles to the cell surface. In neutrophils and monocytes, however, CR3 has been shown to be relatively immobile (Brown et al., 1988). The complement receptors have a low affinity for their ligands, and multiple interactions are usually required for strong adherence of the target cell to the phagocyte (Frank and Fries, 1991). These multiple interactions could be provided by large amounts of C3b deposited on the surface via the classical pathway, which in turn could be enhanced by the high concentration of target GalC on the myelin membrane. *In vivo*

complement activation, and the addition of proinflammatory peptides derived from complement proteins (C3a, C4a, C5a) or a variety of cytokines *in vitro* have been shown to upregulate the complement receptor number and state of activation for phagocytosis (Unkeless and Wright, 1988). Therefore, the activation of early complement components in the vicinity of the target myelin could have effects on macrophage surface receptors, further enhancing the phagocytosis of myelin. This could be tested using soluble anaphylatoxin receptors or antibodies to macrophage surface receptors to inhibit the proposed interaction. As mentioned, reliable quantification of demyelination would ease the interpretation of any results obtained.

The anaphylatoxins C3a, C4a, and C5a are released as cleavage products during activation of the classical pathway of the complement cascade and may act to intensify the inflammatory response and further contribute to the demyelination process (Erdei et al., 1991). C3a, C4a, and C5a increase vascular permeability, have a chemottractive effect on macrophages and other phagocytic cells, increase phagocytic ability of these cells, and, as mentioned, increase the expression of complement receptors CR1 and CR3 on macrophage surfaces (Frank and Fries, 1991). In addition, C3a, C4a, and C5a can elicit macrophages to synthesize or secrete other inflammatory mediators such as interleukin-1 (IL-1) (Schindler et al., 1990) and interleukin-6 (IL-6) (Morgan et al., 1992). The anaphylatoxins may act to attract macrophages to the region of IgG GalC antibody deposition in the demyelination protocol described here. The macrophages could then act to facilitate the removal of myelin, which has been opsonized by early complement cascade proteins. This would be similar to events postulated to underlie EAE, in which macrophage-mediated antibody-dependent responses are shown to occur

(Brosnan et al., 1981; Huitinga et al., 1990). In the C5 deficiency experiments presented here, in which substantial demyelination was observed in the absence of C5, anaphylatoxin C5a was not present, but C3a and C4a could still be acting to trigger local inflammation that could aid the demyelination process. In future experiments that utilize this protocol, the infused reagents could be modified to include cleavage product C5a in order to increase this inflammatory response. Inflammation requires the immigration of phagocytes, and complement-induced phenomena (described above) clearly enhance this process (Carlos and Harlan, 1990) and could contribute to the demyelination described in this work.

Activated microglia/macrophages can act both by phagocytosis through direct contact to disrupt and strip myelin, as in EAE (Lampert and Kies, 1967), and early MS lesions (Prineas and Wright, 1978), and by releasing potentially cytotoxic substances such as proteolytic or lipolytic enzymes (Adams and Hamilton, 1984), free oxygen radicals (Colton and Gilbert, 1987), and inflammatory cytokines such as IL-1 (Giulian et al., 1986), and tumour necrosis factor (TNF) (Matthews, 1981). Cytokines such as TNF $\alpha$  and IL-1 are thought to elicit a complex inflammatory response and have been shown to facilitate myelin destruction (Selmaj and Raine, 1988). In addition, when receptors for C3b and C3bi on macrophages are engaged, IL-1 synthesis is again increased (Bacle et al., 1990; Couturier et al., 1990). TNF $\alpha$  is also believed to act in other demyelinating lesions such as those found in EAE (Kuroda and Shimamoto, 1991) and MS (Sharief and Hentges, 1991), but its exact contribution is unclear. Once the demyelination observed has been triggered by the infused reagents, mononuclear phagocytes may contribute further to the inflammation process through the production of complement components,

including C1-C5, Factor B and D, which they have been observed to produce (Bentley et al., 1978; Whaley, 1980). It is possible that the initial contact between microglia and oligodendrocytes requires complement-receptor binding, but the ultimate fate of the oligodendrocyte/myelin may then be governed by the local release of cytokines (Zajicek et al., 1992). This could be tested by using antibodies to block the binding of the complement fragment C3b to its receptor, thereby preventing the initial contact between microglia and oligodendrocytes and testing the demyelination potential of the cytokines alone.

The activation of other receptors on macrophage surfaces may also contribute to the demyelination observed. Fc receptors may be acting to bind antibody once macrophages are recruited to the region on a larger scale, but not prior to this, since infusion of antibody alone in our current and previous control experiments had no effect on myelin structure (Dyer et al., 1998; Keirstead et al., 1995; Keirstead et al., 1992; Keirstead et al., 1997). Macrophages bind to antibody-coated sheaths via Fc receptors, which will then activate phagocytic responses (Goldenberg et al., 1989). However, studies have shown that when de complemented anti-CNS sera are applied to explant cultures, myelin swelling is observed, but demyelination does not occur even though the myelin is coated with antibody (Johnson et al., 1979). This suggests that complement is a determining factor for macrophage activation, and Fc receptors alone are rather ineffective at inducing demyelination. (Glynn and Linington, 1989). In addition, studies have shown that complement is necessary for maximal myelin phagocytosis by cultured macrophages, even though Fc receptors may be involved in the demyelinating process (DeJong and Smith, 1997). Although Fc receptors could be contributing to the

demyelination observed upon GalC antibody and complement sera infusion, their role appears to be minor, and requires both complement and antibody.

The events postulated above could all occur in the absence of the MAC. In the experimental paradigm presented here, though, the MAC may still contribute to demyelination upon whole complement and antibody infusion, especially in regions where macrophage infiltration is not as apparent. The involvement of the MAC in this case could be further tested using immunolabelling of MAC components, particularly pore-forming C9, to test for their association with myelin. Even when present in sublytic amounts, the MAC could be contributing to demyelination by causing the release of secondary mediators of inflammation such as prostaglandins, leukotrienes, and thromboxane (Daniels et al., 1990; Shin and Carney, 1988). Nevertheless, since the MAC does not appear to be essential for substantial demyelination to occur, in the future it would be interesting to infuse complement deficient in MAC proteins together with GalC antibody to study the effect on regeneration.

In addition to possible contributions to demyelination, the presence of macrophages at lesion sites may be beneficial for axonal regeneration. Studies in lower vertebrates in which CNS axons retain their regenerative ability have shown vigorous phagocytic responses to nerve injury (Dowding et al., 1991; Goodbrand and Gaze, 1991). In nonneuronal tissue, macrophages seem to play an important role in tissue repair after injury (Leibovich and Ross, 1975). However, in the injured CNS, inflammation seems to have a dual nature. It is destructive immediately after injury by contributing to secondary degeneration, but can make many growth-promoting contributions once this damage has been controlled (Hirschberg et al., 1994). Macrophage invasion of damaged white-matter

sites is slower and less extensive in the CNS than in the growth-permissive PNS (Perry et al., 1987). In addition, the macrophages are less efficient at clearing myelin debris in the CNS than in the PNS (George and Griffin, 1994), allowing potentially inhibitory debris to remain for a longer period of time within the CNS. However, the nonpermissive nature of the CNS white matter can be modified in medium conditioned with activated macrophages *in vitro* (David et al., 1990). These findings taken together suggest that activated macrophages are needed at an early stage after injury to promote the healing of the tissue, but late arrival of macrophages may be harmful and could adversely affect the repair process (Schwartz et al., 1999). The importance of the prompt arrival of macrophages to injury regions for effective repair is further supported by experiments using blood macrophages pre-incubated in PNS or CNS tissue and then transferred to the site of spinal cord injuries in rats. These experiments demonstrated that PNS-activated macrophages are more beneficial for axonal regrowth than CNS-activated macrophages (Lazarov-Spiegler et al., 1996), and the regrowth observed with PNS-activated macrophages is correlated with the efficient clearance of myelin from the treated axons and the abundant distribution of the macrophages (Lazarov-Spiegler et al., 1998). Furthermore, macrophage phagocytic activity is inhibited upon exposure to the CNS *in vitro* (Zeev-Brann et al., 1998). Therefore, it might prove advantageous to attract macrophages to a spinal cord injury site at the appropriate time for the treatment of spinal injury.

Macrophages are not the only immune cells that may be beneficial to regeneration. T cells from the adaptive immune response have been shown to accumulate in larger numbers in the injured PNS than in the CNS (Moalem et al., 1999), which has a

propensity for eliminating T cells that is not present in the PNS or in other non-neuronal tissues (Gold et al., 1997). Furthermore, autoimmune T cells specific for MBP have been shown to protect CNS neurons from secondary degeneration after axonal injury (Moalem et al., 1999). Therefore, in addition to macrophages, other immune system cells have been shown to be beneficial to sites of spinal injury.

It has also been suggested that macrophages may promote remyelination. Macrophages have been observed to stimulate myelin formation *in vitro* (Hamilton and Rome, 1994; Loughlin et al., 1997), and inflammation has been shown to be important for remyelination to occur (Graca and Blakemore, 1986). Macrophages have also been shown to express a variety of growth factors and cytokines, including platelet-derived growth factor, epidermal growth factor, transforming growth factor beta, insulin-like growth factor, and nerve growth factor, that have been implicated in such processes as regulation of oligodendrocyte proliferation and survival, myelination, and remyelination (Diemel et al., 1998). Therefore, the presence of macrophages at demyelinated regions of the spinal cord could facilitate the remyelination of these axons.

The findings outlined above suggest that activated macrophages and T cells have the ability to promote CNS healing and regeneration, similar to their repair activity in other organs and tissues, and that the failure of the CNS to regenerate may be partially due to its inability to initiate an appropriate inflammatory response. Perhaps the immunological demyelination protocol described here recruits macrophages and other immune-related cells to spinal cord injury sites at a more opportune time or rate to clear debris, and these macrophages then help facilitate the successful regeneration and remyelination observed in past studies.

In summary, experiments presented here suggest the entire complement cascade is not required for the complement-mediated, GalC antibody-dependent demyelination of the adult rat spinal cord. By infusing serum deficient for one of a group of specific proteins in the complement cascade (C3, C4, Factor B, or C5) together with IgG anti-GalC antibody, I rendered either the classical, alternative, or MAC pathways inactive. Through examination of axonal-myelin ultrastructure, I was then able to determine whether or not the complete complement cascade directly caused the observed myelin changes. I found that upon removal of alternative pathway activity through Factor B deficiency or of MAC activity through C5 deficiency from the infused cocktail, substantial demyelination still occurred. This suggests that the classical pathway alone is sufficient to trigger demyelination when infused with anti-GalC antibody. This demyelination could be occurring through C3b opsonization of target myelin via the classical pathway followed by macrophage infiltration and phagocytosis of myelin, all being promoted by local inflammation induced by complement cleavage products C3a and C4a. Further experiments are needed to verify this mechanism. As the alternative pathway and the MAC do not appear to be crucial for demyelination to occur, refinements of the composition omitting the alternative pathway and MAC proteins could be used to induce demyelination, and would limit potential side effects caused by nonspecific damage that might result from activation of these pathways. Future regeneration experiments could utilize a refined version of the original infusion protocol modified to include only classical pathway proteins C1, C4, C2, and C3, (and possibly C5 to take advantage of the inflammation-inducing abilities of C5a) and IgG anti-GalC, to

achieve substantial focal demyelination of the adult rat spinal cord, thereby removing inhibitory effects of CNS myelin on axonal growth.

The work presented here may prove to be valuable in several areas. First, it adds an important tool to research and clinical science. By determining which of the complement proteins are necessary for demyelination to occur, nonessential proteins can be excluded from the infusion cocktail in future regeneration experiments and clinical testing, thereby limiting any nonspecific damage that these unnecessary proteins may be causing. The result could be an effective, refined demyelination protocol that has been shown to promote axonal regeneration. Second, this technique can be expanded for use as an effective refined biotechnological protocol for general attack of any cell type with an external antigen to which there exists an antibody. Third, the results obtained support the methodology used for elucidating the roles of the various complement components that may be involved in other complement- or immune-mediated phenomena. Finally, the results obtained here could help clarify some of the underlying mechanisms of immune-mediated demyelinating pathologies.

## REFERENCES

- Adams, D. O., and Hamilton, T. A. (1984). The cell biology of macrophage activation. *Annual Review of Immunology* 2, 283-318.
- Ajemian, A., Ness, R., and David, S. (1994). Tenascin in the injured rat optic nerve and in non-neuronal cells in vitro: potential role in neural repair. *Journal of Comparative Neurology* 340, 233-42.
- Alper, C. A., Johnson, A. M., Birtch, A. G., and Moore, F. D. (1969). Human C'3: evidence for the liver as the primary site of synthesis. *Science* 163, 286-8.
- Armstrong, R. C., Harvath, L., and Dubois-Dalcq, M. E. (1990). Type 1 astrocytes and oligodendrocyte-type 2 astrocyte glial progenitors migrate toward distinct molecules. *Journal of Neuroscience Research* 27, 400-7.
- Aydintug, M. K., Leid, R. W., and Widders, P. R. (1990). Antibody enhances killing of *Tritrichomonas foetus* by the alternative bovine complement pathway. *Infection & Immunity* 58, 944-8.
- Bacle, F., Haeffner-Cavaillon, N., Laude, M., Couturier, C., and Kazatchkine, M. D. (1990). Induction of IL-1 release through stimulation of the C3b/C4b complement receptor type one (CR1, CD35) on human monocytes. *Journal of Immunology* 144, 147-52.
- Bansal, R., and Pfeiffer, S. E. (1989). Reversible inhibition of oligodendrocyte progenitor differentiation by a monoclonal antibody against surface galactolipids. *Proceedings of the National Academy of Sciences of the United States of America* 86, 6181-5.
- Bastmeyer, M., Beckmann, M., Schwab, M. E., and Stuermer, C. A. (1991). Growth of regenerating goldfish axons is inhibited by rat oligodendrocytes and CNS myelin but not by goldfish optic nerve tract oligodendrocytelike cells and fish CNS myelin. *Journal of Neuroscience* 11, 626-40.
- Bates, C. A., and Stelzner, D. J. (1993). Extension and regeneration of corticospinal axons after early spinal injury and the maintenance of corticospinal topography. *Experimental Neurology* 123, 106-17.
- Beattie, M. S., Bresnahan, J. C., and Lopate, G. (1990). Metamorphosis alters the response to spinal cord transection in *Xenopus laevis* frogs. *Journal of Neurobiology* 21, 1108-22.
- Benjamins, J. A., Callahan, R. E., Montgomery, I. N., Studzinski, D. M., and Dyer, C. A. (1987). Production and characterization of high titer antibodies to galactocerebroside. *Journal of Neuroimmunology* 14, 325-38.

Bentley, C., Fries, W., and Brade, V. (1978). Synthesis of factors D, B and P of the alternative pathway of complement activation, as well as of C3, by guinea-pig peritoneal macrophages in vitro. *Immunology* 35, 971-80.

Bernstein, J. J. (1967). The regenerative capacity of the telencephalon of the goldfish and rat. *Experimental Neurology* 17, 44-56.

Berry, M. (1982). Post-injury myelin-breakdown products inhibit axonal growth: an hypothesis to explain the failure of axonal regeneration in the mammalian central nervous system. *Bibliotheca Anatomica*, 1-11.

Bhakdi, S., and Trandum-Jensen, J. (1991). Complement lysis: a hole is a hole. *Immunology Today* 12, 318-20; discussion 321.

Bjorklund, A., Katzman, R., Stenevi, U., and West, K. A. (1971). Development and growth of axonal sprouts from noradrenaline and 5-hydroxytryptamine neurones in the rat spinal cord. *Brain Research* 31, 21-33.

Blakemore, W. F. (1984). Limited remyelination of CNS axons by Schwann cells transplanted into the sub-arachnoid space. *Journal of the Neurological Sciences* 64, 265-76.

Blakemore, W. F. (1978). Observations on remyelination in the rabbit spinal cord following demyelination induced by lysolecithin. *Neuropathology & Applied Neurobiology* 4, 47-59.

Blakemore, W. F. (1975). Remyelination by Schwann cells of axons demyelinated by intraspinal injection of 6-aminonicotinamide in the rat. *Journal of Neurocytology* 4, 745-57.

Blakemore, W. F. (1977). Remyelination of CNS axons by Schwann cells transplanted from the sciatic nerve. *Nature* 266, 68-9.

Blakemore, W. F., Eames, R. A., Smith, K. J., and McDonald, W. I. (1977). Remyelination in the spinal cord of the cat following intraspinal injections of lysolecithin. *Journal of the Neurological Sciences* 33, 31-43.

Bornstein, M. B., and Raine, C. S. (1970). Experimental allergic encephalomyelitis. Antiserum inhibition of myelination in vitro. *Laboratory Investigation* 23, 536-42.

Bosio, A., Binczek, E., and Stoffel, W. (1996). Functional breakdown of the lipid bilayer of the myelin membrane in central and peripheral nervous system by disrupted galactocerebroside synthesis. *Proceedings of the National Academy of Sciences of the United States of America* 93, 13280-5.

Braak, E., Griffing, K., Arai, K., Bohl, J., Bratzke, H., and Braak, H. (1999). Neuropathology of Alzheimer's disease: what is new since A. Alzheimer? *European Archives of Psychiatry & Clinical Neuroscience* 249, 14-22.

Bregman, B. S., Kunkel-Bagden, E., McAtee, M., and O'Neill, A. (1989). Extension of the critical period for developmental plasticity of the corticospinal pathway. *Journal of Comparative Neurology* 282, 355-70.

Bregman, B. S., Kunkel-Bagden, E., Schnell, L., Dai, H. N., Gao, D., and Schwab, M. E. (1995). Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. *Nature* 378, 498-501.

Brosnan, C. F., Bornstein, M. B., and Bloom, B. R. (1981). The effects of macrophage depletion on the clinical and pathologic expression of experimental allergic encephalomyelitis. *Journal of Immunology* 126, 614-20.

Brown, D. A., and Rose, J. K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68, 533-44.

Brown, E. J., Bohnsack, J. F., and Gresham, H. D. (1988). Mechanism of inhibition of immunoglobulin G-mediated phagocytosis by monoclonal antibodies that recognize the Mac-1 antigen. *Journal of Clinical Investigation* 81, 365-75.

Brown, E. J., Joiner, K. A., and Frank, M. M. (1983). The role of complement in host resistance to bacteria. *Springer Seminars in Immunopathology* 6, 349-60.

Bruck, W., and Friede, R. L. (1990). Anti-macrophage CR3 antibody blocks myelin phagocytosis by macrophages in vitro. *Acta Neuropathologica* 80, 415-8.

Burger, R., Gordon, J., Stevenson, G., Ramadori, G., Zanker, B., Hadding, U., and Bitter-Suermann, D. (1986). An inherited deficiency of the third component of complement, C3, in guinea pigs. *European Journal of Immunology* 16, 7-11.

Burton, D. R. (1990). The conformation of antibodies. In *Fc receptors and the action of antibodies.*, H. Metzger, ed. (Washington, D.C.: Raven Press), pp. 31-54.

Cadelli, D., and Schwab, M. E. (1991). Regeneration of lesioned septohippocampal acetylcholinesterase-positive axons improved by antibodies against the myelin-associated neurite growth inhibitors NI-35/250. *European Journal of Neuroscience* 3, 825-832.

Carlos, T. M., and Harlan, J. M. (1990). Membrane proteins involved in phagocyte adherence to endothelium. *Immunological Reviews* 114, 5-28.

Caroni, P., Savio, T., and Schwab, M. E. (1988a). Central nervous system regeneration: oligodendrocytes and myelin as non-permissive substrates for neurite growth. *Progress in Brain Research* 78, 363-70.

Caroni, P., and Schwab, M. E. (1988b). Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron* 1, 85-96.

Caroni, P., and Schwab, M. E. (1988c). Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *Journal of Cell Biology* 106, 1281-8.

Carroll, W. M., Jennings, A. R., and Mastaglia, F. L. (1984). Experimental demyelinating optic neuropathy induced by intra-neural injection of galactocerebroside antiserum. *Journal of the Neurological Sciences* 65, 125-35.

Chan, A. R., Karp, D. R., Shreffler, D. C., and Atkinson, J. P. (1984). The 20 faces of the fourth component of complement. *Immunology Today* 5, 200-203.

Chen, M. S., Huber, A. B., van der Haar, M. E., Frank, M., Schnell, L., Spillmann, A. A., Christ, F., and Schwab, M. E. (2000). Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 403, 434-9.

Cheng, H., Cao, Y., and Olson, L. (1996). Spinal cord repair in adult paraplegic rats: Partial restoration of hind limb function. *Science* 273, 510-513.

Clarke, J. D., Alexander, R., and Holder, N. (1988). Regeneration of descending axons in the spinal cord of the axolotl. *Neuroscience Letters* 89, 1-6.

Coetzee, T., Fujita, N., Dupree, J., Shi, R., Blight, A., Suzuki, K., and Popko, B. (1996). Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. *Cell* 86, 209-19.

Cohen, A. H., Mackler, S. A., and Selzer, M. E. (1988). Behavioral recovery following spinal transection: functional regeneration in the lamprey CNS. *Trends in Neurosciences* 11, 227-31.

Colton, C. A., and Gilbert, D. L. (1987). Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Letters* 223, 284-8.

Compston, D. A., Morgan, B. P., Campbell, A. K., Wilkins, P., Cole, G., Thomas, N. D., and Jasani, B. (1989). Immunocytochemical localization of the terminal complement complex in multiple sclerosis. *Neuropathology & Applied Neurobiology* 15, 307-16.

Couturier, C., Haeffner-Cavaillon, N., Weiss, L., Fischer, E., and Kazatchkine, M. D. (1990). Induction of cell-associated interleukin 1 through stimulation of the adhesion-promoting proteins LFA-1 (CD11a/CD18) and CR3 (CD11b/CD18) of human monocytes. *European Journal of Immunology* 20, 999-1005.

Crutcher, K. A. (1989). Tissue sections from the mature rat brain and spinal cord as substrates for neurite outgrowth in vitro: extensive growth on gray matter but little growth on white matter. *Experimental Neurology* 104, 39-54.

Cui, L., Carney, D. F., and Hugli, T. E. (1994). Primary structure and functional characterization of rat C5a: an anaphylatoxin with unusually high potency. *Protein Science* 3, 1169-77.

Dal Canto, M. C., and Lipton, H. L. (1980). Schwann cell remyelination and recurrent demyelination in the central nervous system of mice infected with attenuated Theiler's virus. *American Journal of Pathology* 98, 101-22.

Daniels, R. H., Williams, B. D., and Morgan, B. P. (1990). Human rheumatoid synovial cell stimulation by the membrane attack complex and other pore-forming toxins in vitro: the role of calcium in cell activation. *Immunology* 71, 312-6.

David, S., and Aguayo, A. J. (1981). Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science* 214, 931-3.

David, S., Bouchard, C., Tstas, O., and Giftchristos, N. (1990). Macrophages can modify the nonpermissive nature of the adult mammalian central nervous system. *Neuron* 5, 463-469.

Davis, B. M., Ayers, J. L., Koran, L., Carlson, J., Anderson, M. C., and Simpson, S. B., Jr. (1990). Time course of salamander spinal cord regeneration and recovery of swimming: HRP retrograde pathway tracing and kinematic analysis. *Experimental Neurology* 108, 198-213.

Davis, B. M., Duffy, M. T., and Simpson, S. B., Jr. (1989). Bulbospinal and intraspinal connections in normal and regenerated salamander spinal cord. *Experimental Neurology* 103, 41-51.

Davis, G. R., Jr., and McClellan, A. D. (1994a). Extent and time course of restoration of descending brainstem projections in spinal cord-transected lamprey. *Journal of Comparative Neurology* 344, 65-82.

Davis, G. R., Jr., and McClellan, A. D. (1994b). Long distance axonal regeneration of identified lamprey reticulospinal neurons. *Experimental Neurology* 127, 94-105.

DeJong, B. A., and Smith, M. E. (1997). A role for complement in phagocytosis of myelin. *Neurochemical Research* 22, 491-8.

Devine, D. V. (1991). The regulation of complement on cell surfaces. *Transfusion Medicine Reviews* 5, 123-131.

Diemel, L. T., Copelman, C. A., and Cuzner, M. L. (1998). Macrophages in CNS remyelination: friend or foe? *Neurochemical Research* 23, 341-7.

Dorfman, S. H., Fry, J. M., and Silberberg, D. H. (1979). Antiserum induced myelination inhibition in vitro without complement. *Brain Research* 177, 105-14.

Dow, K. E., Ethell, D. W., Steeves, J. D., and Riopelle, R. J. (1994). Molecular correlates of spinal cord repair in the embryonic chick: heparan sulfate and chondroitin sulfate proteoglycans. *Experimental Neurology* 128, 233-8.

Dowding, A. J., Maggs, A., and Scholes, J. (1991). Diversity amongst the microglia in growing and regenerating fish CNS: immunohistochemical characterization using FL.1, an anti-macrophage monoclonal antibody. *Glia* 4, 345-64.

Dubois-Dalcq, M., Niedieck, B., and Buyse, M. (1970). Action of anti-cerebroside sera on myelinated nervous tissue cultures. *Pathologia Europaea* 5, 331-47.

Dyer, C. A., and Benjamins, J. A. (1988). Antibody to galactocerebroside alters organization of oligodendroglial membrane sheets in culture. *Journal of Neuroscience* 8, 4307-18.

Dyer, C. A., and Benjamins, J. A. (1990). Glycolipids and transmembrane signaling: antibodies to galactocerebroside cause an influx of calcium in oligodendrocytes. *Journal of Cell Biology* 111, 625-33.

Dyer, J. K., Bourque, J. A., and Steeves, J. D. (1998). Regeneration of brainstem-spinal axons after lesion and immunological disruption of myelin in adult rat. *Experimental Neurology* 154, 12-22.

Dyer, J. K., Bourque, J. A., van Rooijen, N., and Steeves, J. D. (1999). Myelin removal during immunological myelin suppression is mediated by blood-derived macrophages. The Eighth International Symposium on Neural Regeneration Abstracts, In *Experimental Neurology* *In press*.

Eames, R. A., Jacobson, S. G., and McDonald, W. I. (1977). Pathological changes in the optic chiasm of the cat following local injection of diphtheria toxin. *Journal of the Neurological Sciences* 32, 381-93.

Eikelenboom, P., Hack, C. E., Rozemuller, J. M., and Stam, F. C. (1989). Complement activation in amyloid plaques in Alzheimer's dementia. *Virchows Archiv. B, Cell Pathology Including Molecular Pathology* 56, 259-62.

Eikelenboom, P., and Stam, F. C. (1982). Immunoglobulins and complement factors in senile plaques. An immunoperoxidase study. *Acta Neuropathologica* 57, 239-42.

Erdei, A., Fust, G., and Gergely, J. (1991). The role of C3 in the immune response. *Immunology Today* 12, 332-7.

Forehand, C. J., and Farel, P. B. (1982). Anatomical and behavioral recovery from the effects of spinal cord transection: dependence on metamorphosis in anuran larvae. *Journal of Neuroscience* 2, 654-52.

Frank, M. M., and Fries, L. F. (1991). The role of complement in inflammation and phagocytosis. *Immunology Today* 12, 322-6.

Fruttiger, M., Schachner, M., and Martini, R. (1995). Tenascin-C expression during wallerian degeneration in C57BL/Wlds mice: possible implications for axonal regeneration. *Journal of Neurocytology* 24, 1-14.

George, R., and Griffin, J. W. (1994). Delayed macrophage responses and myelin clearance during Wallerian degeneration in the central nervous system: the dorsal radicotomy model. *Experimental Neurology* 129, 225-36.

Gerard, C., and Gerard, N. P. (1994). C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annual Review of Immunology* 12, 775-808.

Giulian, D., Baker, T. J., Shih, L. C., and Lachman, L. B. (1986). Interleukin 1 of the central nervous system is produced by ameboid microglia. *Journal of Experimental Medicine* 164, 594-604.

Glynn, P., and Linington, C. (1989). Cellular and molecular mechanisms of autoimmune demyelination in the central nervous system. *Critical Reviews in Neurobiology* 4, 367-85.

Gold, R., Hartung, H. P., and Lassmann, H. (1997). T-cell apoptosis in autoimmune diseases: termination of inflammation in the nervous system and other sites with specialized immune-defense mechanisms. *Trends in Neurosciences* 20, 399-404.

Goldenberg, P. Z., Kwon, E. E., Benjamins, J. A., Whitaker, J. N., Quarles, R. H., and Prineas, J. W. (1989). Opsonization of normal myelin by anti-myelin antibodies and normal serum. *Journal of Neuroimmunology* 23, 157-66.

Goodbrand, I. A., and Gaze, R. M. (1991). Microglia in tadpoles of *Xenopus laevis*: normal distribution and the response to optic nerve injury. *Anatomy & Embryology* 184, 71-82.

Gotz, B., Scholze, A., Clement, A., Joester, A., Schutte, K., Wigger, F., Frank, R., Spiess, E., Ekblom, P., and Faissner, A. (1996). Tenascin-C contains distinct adhesive, anti-adhesive, and neurite outgrowth promoting sites for neurons. *Journal of Cell Biology* 132, 681-99.

Graca, D. L., and Blakemore, W. F. (1986). Delayed remyelination in rat spinal cord following ethidium bromide injection. *Neuropathology & Applied Neurobiology* 12, 593-605.

Grant, S., and Keating, M. J. (1986). Ocular migration and the metamorphic and postmetamorphic maturation of the retinotectal system in *Xenopus laevis*: an autoradiographic and morphometric study. *Journal of Embryology & Experimental Morphology* 92, 43-69.

Griffin, F. M., Jr., and Griffin, J. A. (1980). Augmentation of macrophage complement receptor function in vitro. II. Characterization of the effects of a unique lymphokine upon the phagocytic capabilities of macrophages. *Journal of Immunology* 125, 844-9.

Hamilton, S. P., and Rome, L. H. (1994). Stimulation of in vitro myelin synthesis by microglia. *Glia* 11, 326-35.

Hardy, J., and Allsop, D. (1991). Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends in Pharmacological Sciences* 12, 383-8.

Hartmann, K., Henz, B. M., Kruger-Krasagakes, S., Kohl, J., Burger, R., Guhl, S., Haase, I., Lippert, U., and Zuberbier, T. (1997). C3a and C5a stimulate chemotaxis of human mast cells. *Blood* 89, 2863-70.

Hartung, H. P., Schwenke, C., Bitter-Suermann, D., and Toyka, K. V. (1987). Guillain-Barre syndrome: activated complement components C3a and C5a in CSF. *Neurology* 37, 1006-9.

Harvey, B. S., Baker, C. J., and Edwards, M. S. (1992). Contributions of complement and immunoglobulin to neutrophil-mediated killing of enterococci. *Infection & Immunity* 60, 3635-40.

Hasan, S. J., Keirstead, H. S., Muir, G. D., and Steeves, J. D. (1993). Axonal regeneration contributes to repair of injured brainstem-spinal neurons in embryonic chick. *Journal of Neuroscience* 13, 492-507.

Hays, A. P., Lee, S. S., and Latov, N. (1988). Immune reactive C3d on the surface of myelin sheaths in neuropathy. *Journal of Neuroimmunology* 18, 231-44.

Hirschberg, D. L., Yoles, E., Belkin, M., and Schwartz, M. (1994). Inflammation after axonal injury has conflicting consequences for recovery of function: rescue of spared axons is impaired but regeneration is supported [see comments]. *Journal of Neuroimmunology* 50, 9-16.

Hoover, D. L., Berger, M., Hammer, C. H., and Meltzer, M. S. (1985). Complement-mediated serum cytotoxicity for *Leishmania major* amastigotes: killing by serum deficient in early components of the membrane attack complex. *Journal of Immunology* 135, 570-4.

Horwitz, M. A. (1982). Phagocytosis of microorganisms. *Reviews of Infectious Diseases* 4, 104-23.

Huey, R., and Hugli, T. E. (1985). Characterization of a C5a receptor on human polymorphonuclear leukocytes (PMN). *Journal of Immunology* 135, 2063-8.

Hugli, T. E., Gerard, C., Kawahara, M., Scheetz, M. E. d., Barton, R., Briggs, S., Koppel, G., and Russell, S. (1981). Isolation of three separate anaphylatoxins from complement-activated human serum. *Molecular & Cellular Biochemistry* 41, 59-66.

Huitinga, I., van Rooijen, N., de Groot, C. J., Uitdehaag, B. M., and Dijkstra, C. D. (1990). Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *Journal of Experimental Medicine* 172, 1025-33.

Janeway Jr., C. A., and Travers, P. (1997). *Immunobiology: The Immune System in Health and Disease, Third Edition* (London, San Francisco, New York: Current Biology Limited, Garland Publishing Inc.).

Jhaveri, S., Erzurumlu, R. S., Friedman, B., and Schneider, G. E. (1992). Oligodendrocytes and myelin formation along the optic tract of the developing hamster: an immunohistochemical study using the Rip antibody. *Glia* 6, 138-48.

Johnson, A. B., Raine, C. S., and Bornstein, M. B. (1979). Experimental allergic encephalomyelitis: serum immunoglobulin binds to myelin and oligodendrocytes in cultured tissue: ultrastructural-immunoperoxidase observations. *Laboratory Investigation* 40, 568-75.

Kalil, K., and Reh, T. (1982). A light and electron microscopic study of regrowing pyramidal tract fibers. *Journal of Comparative Neurology* 211, 265-75.

Kapfhammer, J. P., and Schwab, M. E. (1994). Inverse patterns of myelination and GAP\_43 expression in the adult CNS: Neurite growth inhibitors as regulators of neuronal plasticity? *The Journal of Comparative Neurology* 340, 194-206.

Keifer, J., and Kalil, K. (1991). Effects of infant versus adult pyramidal tract lesions on locomotor behavior in hamsters. *Experimental Neurology* 111, 98-105.

Keirstead, H. S., Dyer, J. K., Sholomenko, G. N., McGraw, J., Delaney, K. R., and Steeves, J. D. (1995). Axonal regeneration and physiological activity following transection and immunological disruption of myelin within the hatchling chick spinal cord. *Journal of Neuroscience* 15, 6963-74.

Keirstead, H. S., Hasan, S. J., Muir, G. D., and Steeves, J. D. (1992). Suppression of the onset of myelination extends the permissive period for the functional repair of embryonic spinal cord. *Proc.Natl.Acad.Sci.* 89, 11664-11668.

Keirstead, H. S., Hughes, H. C., and Blakemore, W. F. (1998a). A quantifiable model of axonal regeneration in the demyelinated adult rat spinal cord. *Experimental Neurology* 151, 303-13.

Keirstead, H. S., Levine, J. M., and Blakemore, W. F. (1998b). Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord. *Glia* 22, 161-70.

Keirstead, H. S., Morgan, S. V., Wilby, M. J., and Fawcett, J. W. (1999). Enhanced axonal regeneration following combined demyelination plus schwann cell transplantation therapy in the injured adult spinal cord. *Experimental Neurology* 159, 225-36.

Keirstead, H. S., Pataky, D. M., McGraw, J., and Steeves, J. D. (1997). In vivo immunological suppression of spinal cord myelin development. *Brain Research Bulletin* 44, 727-34.

Kochi, S. K., Johnson, R. C., and Dalmaso, A. P. (1993). Facilitation of complement-dependent killing of the Lyme disease spirochete, *Borrelia burgdorferi*, by specific immunoglobulin G Fab antibody fragments. *Infection & Immunity* 61, 2532-6.

Kolb, W. P., Kolb, L. M., and Podack, E. R. (1979). C1q: isolation from human serum in high yield by affinity chromatography and development of a highly sensitive hemolytic assay. *Journal of Immunology* 122, 2103-11.

Kolodkin, A. L., Matthes, D. J., and Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* 75, 1389-99.

Koshy, K. M., and Boggs, J. M. (1996). Investigation of the calcium-mediated association between the carbohydrate head groups of galactosylceramide and galactosylceramide I3 sulfate by electrospray ionization mass spectrometry. *Journal of Biological Chemistry* 271, 3496-9.

Koski, C. L. (1990). Characterization of complement-fixing antibodies to peripheral nerve myelin in Guillain-Barre syndrome. *Annals of Neurology* 27, S44-7.

Koski, C. L., Gratz, E., Sutherland, J., and Mayer, R. F. (1986). Clinical correlation with anti-peripheral-nerve myelin antibodies in Guillain-Barre syndrome. *Annals of Neurology* 19, 573-7.

Koski, C. L., Sanders, M. E., Swoveland, P. T., Lawley, T. J., Shin, M. L., Frank, M. M., and Joiner, K. A. (1987). Activation of terminal components of complement in patients with Guillain-Barre syndrome and other demyelinating neuropathies. *Journal of Clinical Investigation* 80, 1492-7.

Koynova, R., and Caffrey, M. (1995). Phases and phase transitions of the sphingolipids. *Biochimica et Biophysica Acta* 1255, 213-36.

Kramer, E. M., Koch, T., Niehaus, A., and Trotter, J. (1997). Oligodendrocytes direct glycosyl phosphatidylinositol-anchored proteins to the myelin sheath in glycosphingolipid-rich complexes. *Journal of Biological Chemistry* 272, 8937-45.

Kubba, R., Al-Gindan, Y., el-Hassan, A. M., and Omer, A. H. (1987). Clinical diagnosis of cutaneous leishmaniasis (oriental sore). *Journal of the American Academy of Dermatology* 16, 1183-9.

Kuroda, Y., and Shimamoto, Y. (1991). Human tumor necrosis factor-alpha augments experimental allergic encephalomyelitis in rats. *Journal of Neuroimmunology* 34, 159-64.

Lampert, P. W., and Kies, M. W. (1967). Mechanism of demyelination in allergic encephalomyelitis of guinea pigs. An electron microscopic study. *Experimental Neurology* 18, 210-23.

Lang, D. M., Rubin, B. P., Schwab, M. E., and Stuermer, C. A. O. (1995). CNS myelin and oligodendrocytes of the *Xenopus* spinal cord - but not optic nerve - are nonpermissive for axon growth. *Journal of Neuroscience* 15, 99-109.

Law, S. K. A., and Reid, K. B. (1989). *Complement*, D. Male and D. Rickwood, eds. (Washington, D.C.: IRL Press, Oxford).

Laywell, E. D., Dorries, U., Bartsch, U., Faissner, A., Schachner, M., and Steindler, D. A. (1992). Enhanced expression of the developmentally regulated extracellular matrix molecule tenascin following adult brain injury. *Proceedings of the National Academy of Sciences of the United States of America* 89, 2634-8.

Lazarov-Spiegler, O., Solomon, A. S., and Schwartz, M. (1998). Peripheral Nerve-Stimulated Macrophages Simulate a Peripheral Nerve-Like Regenerative Response in Rat Transected Optic Nerve. *Glia* 24, 329-337.

Lazarov-Spiegler, O., Solomon, A. S., Zeev-Brann, A. B., Hirschberg, D. L., Lavie, V., and Schwartz, M. (1996). Transplantation of activated macrophages overcomes central nervous system regrowth failure. *FASEB Journal* 10, 1296-302.

Leibovich, S. J., and Ross, R. (1975). The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *American Journal of Pathology* 78, 71-100.

Levine, J. M. (1994). Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury. *Journal of Neuroscience* 14, 4716-30.

Li, Y., Field, P. M., and Raisman, G. (1997). Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells. *Science* 277, 2000-2002.

Li, Y., and Raisman, G. (1994). Schwann cells induce sprouting in motor and sensory axons in the adult rat spinal cord. *Journal of Neuroscience* 14, 4050-63.

Linington, C., Lassmann, H., Morgan, B. P., and Compston, D. A. (1989). Immunohistochemical localisation of terminal complement component C9 in experimental allergic encephalomyelitis. *Acta Neuropathologica* 79, 78-85.

Linington, C., Morgan, B. P., Scolding, N. J., Wilkins, P., Piddlesden, S., and Compston, D. A. (1989). The role of complement in the pathogenesis of experimental allergic encephalomyelitis. *Brain* 112, 895-911.

Loughlin, A. J., Copelman, C. A., Hall, A., Armer, T., Young, B. C., Landon, D. N., and Cuzner, M. L. (1997). Myelination and remyelination of aggregate rat brain cell cultures enriched with macrophages. *Journal of Neuroscience Research* 47, 384-92.

Ludwin, S. K. (1978). Central nervous system demyelination and remyelination in the mouse: an ultrastructural study of cuprizone toxicity. *Laboratory Investigation* 39, 597-612.

Lunn, K. F., Fanarraga, M. L., and Duncan, I. D. (1995). Myelin mutants: new models and new observations. *Microscopy Research & Technique* 32, 183-203.

Luo, Y., Raible, D., and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* 75, 217-27.

Lurie, D. I., Pijak, D. S., and Selzer, M. E. (1994). Structure of reticulospinal axon growth cones and their cellular environment during regeneration in the lamprey spinal cord. *Journal of Comparative Neurology* 344, 559-80.

Lurie, D. I., and Selzer, M. E. (1991). Axonal regeneration in the adult lamprey spinal cord. *Journal of Comparative Neurology* 306, 409-16.

Mastaglia, F. L., Carroll, W. M., and Jennings, A. R. (1989). Spinal cord lesions induced by antigalactocerebroside serum. *Clinical & Experimental Neurology* 26, 33-44.

Matthews, N. (1981). Production of an anti-tumour cytotoxin by human monocytes. *Immunology* 44, 135-42.

Mayer, M. M. (1972). Mechanism of cytolysis by complement. *Proceedings of the National Academy of Sciences of the United States of America* 69, 2954-8.

McGeer, P. L., Akiyama, H., Itagaki, S., and McGeer, E. G. (1989). Activation of the classical complement pathway in brain tissue of Alzheimer patients. *Neuroscience Letters* 107, 341-6.

McGeer, P. L., Akiyama, H., Itagaki, S., and McGeer, E. G. (1989). Immune system response in Alzheimer's disease. *Canadian Journal of Neurological Sciences* 16, 516-27.

McGeer, P. L., and McGeer, E. G. (1992). Complement proteins and complement inhibitors in Alzheimer's disease. *Research in Immunology* 143, 621-4.

McKeon, R. J., Schreiber, R. C., Rudge, J. S., and Silver, J. (1991). Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *Journal of Neuroscience* 11(11), 3398-3411.

McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J., and Braun, P. E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* 13, 805-811.

Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I. R., and Schwartz, M. (1999). Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nature Medicine* 5, 49-55.

Moalem, G., Monsonego, A., Shani, Y., Cohen, I. R., and Schwartz, M. (1999). Differential T cell response in central and peripheral nerve injury: connection with immune privilege. *FASEB Journal* 13, 1207-17.

Mollnes, T. E., Vandvik, B., Lea, T., and Vartdal, F. (1987). Intrathecal complement activation in neurological diseases evaluated by analysis of the terminal complement complex. *Journal of the Neurological Sciences* 78, 17-28.

Morell, P., Quarles, R. H., and Norton, W. T. (1994). Myelin formation, structure, and biochemistry. In *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects.*, G. J. Siegel, ed. (New York: Raven Press Ltd.), pp. 117-144.

Morgan, B. P., and Gasque, P. (1997). Extrahepatic complement biosynthesis: where, when and why? *Clinical & Experimental Immunology* 107, 1-7.

Morgan, E. L., Sanderson, S., Scholz, W., Noonan, D. J., Weigle, W. O., and Hugli, T. E. (1992). Identification and characterization of the effector region within human C5a responsible for stimulation of IL-6 synthesis. *Journal of Immunology* 148, 3937-42.

Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R., and Filbin, M. T. (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* 13, 757-67.

Newman, S. L., and Tucci, M. A. (1990). Regulation of human monocyte/macrophage function by extracellular matrix. Adherence of monocytes to collagen matrices enhances phagocytosis of opsonized bacteria by activation of complement receptors and enhancement of Fc receptor function. *Journal of Clinical Investigation* 86, 703-14.

Noel, G. J., Katz, S., and Edelson, P. J. (1988). Complement-mediated early clearance of *Haemophilus influenzae* type b from blood is independent of serum lytic activity. *Journal of Infectious Diseases* 157, 85-90.

Nolte, C., Moller, T., Walter, T., and Kettenmann, H. (1996). Complement 5a controls motility of murine microglial cells in vitro via activation of an inhibitory G-protein and the rearrangement of the actin cytoskeleton. *Neuroscience* 73, 1091-107.

Norton, W. T., and Cammer, W. (1984). Isolation and characterization of myelin. In *Myelin.*, P. Morell, ed. (New York: Plenum Press), pp. 147-195.

Ohta, H., Yoshikawa, Y., Kai, C., Yamanouchi, K., and Okada, H. (1984). Lysis of horse red blood cells mediated by antibody-independent activation of the alternative pathway of chicken complement. *Immunology* 52, 437-44.

Oohira, A., Matsui, F., and Katoh-Semba, R. (1991). Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC12D cells. *Journal of Neuroscience* 11, 822-7.

Pabst, H., Day, N. K., Gewurz, H., and Good, R. A. (1971). Prevention of experimental allergic encephalomyelitis with cobra venom factor. *Proceedings of the Society for Experimental Biology & Medicine* 136, 555-60.

Paino, C. L., Fernandez-Valle, C., Bates, M. L., and Bunge, M. B. (1994). Regrowth of axons in lesioned adult rat spinal cord: promotion by implants of cultured Schwann cells. *Journal of Neurocytology* 23, 433-52.

Pepys, M. B. (1974). Role of complement in induction of antibody production in vivo. Effect of cobra factor and other C3-reactive agents on thymus-dependent and thymus-independent antibody responses. *Journal of Experimental Medicine* 140, 126-45.

Perry, V. H., Andersson, P. B., and Gordon, S. (1993). Macrophages and inflammation in the central nervous system. *Trends in Neurosciences* 16, 268-73.

Perry, V. H., Brown, M. C., and Gordon, S. (1987). The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. *Journal of Experimental Medicine* 165, 1218-23.

Pesheva, P., Spiess, E., and Schachner, M. (1989). J1-160 and J1-180 are oligodendrocyte-secreted nonpermissive substrates for cell adhesion. *Journal of Cell Biology* 109, 1765-78.

Peters, A., Webster, H., and Palay, S. L. (1976). *The Fine Structure of the Nervous System: the Neurons and Supporting Cells* (Philadelphia: Saunders).

Piddlesden, S., Lassmann, H., Laffafian, I., Morgan, B. P., and Linington, C. (1991). Antibody-mediated demyelination in experimental allergic encephalomyelitis is independent of complement membrane attack complex formation. *Clinical & Experimental Immunology* 83, 245-50.

Piddlesden, S. J., Storch, M. K., Hibbs, M., Freeman, A. M., Lassmann, H., and Morgan, B. P. (1994). Soluble recombinant complement receptor 1 inhibits inflammation and demyelination in antibody-mediated demyelinating experimental allergic encephalomyelitis. *Journal of Immunology* 152, 5477-84.

Pindzola, R. R., Doller, C., and Silver, J. (1993). Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. *Developmental Biology (Orlando)* 156, 34-48.

Poltorak, M., Sadoul, R., Keilhauer, G., Landa, C., Fahrig, T., and Schachner, M. (1987). Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interaction. *Journal of Cell Biology* 105, 1893-9.

Prineas, J. W., and Wright, R. G. (1978). Macrophages, lymphocytes, and plasma cells in the perivascular compartment in chronic multiple sclerosis. *Laboratory Investigation* 38, 409-21.

Quarles, R. H., Farrer, R. G., and Yim, S. H. (1997). Structure and Function of Myelin, an Extended and Biochemically Modified Cell Surface Membrane. In *Cell Biology and Pathology of Myelin: Evolving Biological Concepts and Therapeutic Approaches*, R. H. Juurlink, R. M. Devon, J. R. Doucette, A. J. Nazarali, D. J. Schreyer and V. M. K. Verge, eds. (New York, London: Plenum Press), pp. 1-12.

Raine, C. S., and Bornstein, M. B. (1970a). Experimental allergic encephalomyelitis: a light and electron microscope study of remyelination and "sclerosis" in vitro. *Journal of Neuropathology & Experimental Neurology* 29, 552-74.

Raine, C. S., and Bornstein, M. B. (1970b). Experimental allergic encephalomyelitis: an ultrastructural study of experimental demyelination in vitro. *Journal of Neuropathology & Experimental Neurology* 29, 177-91.

Ramon Y Cajal, S. (1928). *Degeneration and Regeneration of the Nervous System* (New York: Hafner).

Ramon-Cueto, A., Cordero, M. I., Santos-Benito, F. F., and Avila, J. (2000). Functional Recovery of Paraplegic Rats and Motor Axon Regeneration in Their Spinal Cords by Olfactory Ensheathing Glia. *Neuron* 25, 425-435.

Ranscht, B., Clapshaw, P. A., Price, J., Noble, M., and Seifert, W. (1982). Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proceedings of the National Academy of Sciences of the United States of America* 79, 2709-13.

Reh, T., and Kalil, K. (1982). Functional role of regrowing pyramidal tract fibers. *Journal of Comparative Neurology* 211, 276-83.

Richardson, P. M., McGuinness, U. M., and Aguayo, A. J. (1980). Axons from CNS neurons regenerate into PNS grafts. *Nature* 284, 264-5.

Ross, G. D., and Medof, M. E. (1985). Membrane complement receptors specific for bound fragments of C3. *Advances in Immunology* 37, 217-67.

Rother, K. (1988). The manipulation of complement in vivo. In *The Complement System*, K. Rother and G. O. Till, eds. (Berlin: Springer-Verlag), pp. 444-451.

Saunders, N. R., Balkwill, P., Knott, G., Habgood, M. D., Mollgard, K., Treherne, J. M., and Nicholls, J. G. (1992). Growth of axons through a lesion in the intact CNS of fetal rat maintained in long-term culture. *Proceedings of the Royal Society of London - Series B: Biological Sciences* 250, 171-80.

Savio, T., and Schwab, M. E. (1990). Lesioned corticospinal tract axons regenerate in myelin-free rat spinal cord. *Proceedings of the National Academy of Sciences of the United States of America* 87, 4130-3.

Schaber, B., Bruchelt, G., Meyle, J., Jeschke, B., Handgretinger, R., Niethammer, D., Mayer, P., Rassner, G., and Fierlbeck, G. (1994). Chemotactic activity of substances derived from antibody-loaded tumor cells on granulocytes. *Immunology Letters* 41, 67-71.

Schindler, R., Gelfand, J. A., and Dinarello, C. A. (1990). Recombinant C5a stimulates transcription rather than translation of interleukin-1 (IL-1) and tumor necrosis factor: translational signal provided by lipopolysaccharide or IL-1 itself. *Blood* 76, 1631-8.

Schnell, L., and Schwab, M. E. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature* 343, 269-72.

Schnell, L., and Schwab, M. E. (1993). Sprouting and regeneration of lesioned corticospinal tract fibres in the adult rat spinal cord. *European Journal of Neuroscience* 5, 1156-71.

Schreyer, D. J., and Jones, E. G. (1983). Growing corticospinal axons by-pass lesions of neonatal rat spinal cord. *Neuroscience* 9, 31-40.

Schreyer, D. J., and Jones, E. G. (1982). Growth and target finding by axons of the corticospinal tract in prenatal and postnatal rats. *Neuroscience* 7, 1837-53.

Schulman, E. S., Post, T. J., Henson, P. M., and Giclas, P. C. (1988). Differential effects of the complement peptides, C5a and C5a des Arg on human basophil and lung mast cell histamine release. *Journal of Clinical Investigation* 81, 918-23.

Schwab, C., Steele, J. C., and McGeer, P. L. (1996). Neurofibrillary tangles of Guam parkinson-dementia are associated with reactive microglia and complement proteins. *Brain Research* 707, 196-205.

Schwab, M. E., and Caroni, P. (1988). Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. *Journal of Neuroscience* 8, 2381-93.

Schwartz, M., Moalem, G., Leibowitz-Amit, R., and Cohen, I. R. (1999). Innate and adaptive immune responses can be beneficial for CNS repair. *Trends in Neurosciences* 22, 295-299.

Scolding, N. J., and Compston, D. A. (1991). Oligodendrocyte-macrophage interactions in vitro triggered by specific antibodies. *Immunology* 72, 127-32.

Selmaj, K. W., and Raine, C. S. (1988). Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Annals of Neurology* 23, 339-46.

Sergott, R. C., Brown, M. J., Silberberg, D. H., and Lisak, R. P. (1984). Antigalactocerebroside serum demyelinates optic nerve in vivo. *Journal of the Neurological Sciences* 64, 297-303.

Sharief, M. K., and Hentges, R. (1991). Association between tumor necrosis factor-alpha and disease progression in patients with multiple sclerosis [see comments]. *New England Journal of Medicine* 325, 467-72.

Sharma, S. C., Jadhao, A. G., and Rao, P. D. (1993). Regeneration of supraspinal projection neurons in the adult goldfish. *Brain Research* 620, 221-8.

Shimizu, I., Oppenheim, R. W., O'Brien, M., and Shneiderman, A. (1990). Anatomical and functional recovery following spinal cord transection in the chick embryo. *Journal of Neurobiology* 21, 918-37.

Shin, M. L., and Carney, D. F. (1988). Cytotoxic action and other metabolic consequences of terminal complement proteins. *Progress in Allergy* 40, 44-81.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. *Nature* 387, 569-72.

Sims, T. J., Gilmore, S. A., Waxman, S. G., and Klinge, E. (1985). Dorsal-ventral differences in the glia limitans of the spinal cord: an ultrastructural study in developing normal and irradiated rats. *Journal of Neuropathology & Experimental Neurology* 44, 415-29.

Singhrao, S. K., Neal, J. W., Gasque, P., Morgan, B. P., and Newman, G. R. (1996). Role of complement in the aetiology of Pick's disease? *Journal of Neuropathology & Experimental Neurology* 55, 578-93.

Sivron, T., Schwab, M. E., and Schwartz, M. (1994). Presence of growth inhibitors in fish optic nerve myelin: postinjury changes. *Journal of Comparative Neurology* 343, 237-46.

Snow, D. M., Lemmon, V., Carrino, D. A., Caplan, A. I., and Silver, J. (1990). Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Experimental Neurology* 109, 111-30.

Spillmann, A. A., Amberger, V. R., and Schwab, M. E. (1997). High molecular weight protein of human central nervous system myelin inhibits neurite outgrowth: an effect which can be neutralized by the monoclonal antibody IN-1. *European Journal of Neuroscience* 9, 549-55.

Spillmann, A. A., Bandtlow, C. E., Lottspeich, F., Keller, F., and Schwab, M. E. (1998). Identification and characterization of a bovine neurite growth inhibitor (bNI-220). *Journal of Biological Chemistry* 273, 19283-93.

Steeves, J. D., Keirstead, H. S., Ethell, D. W., Hasan, S. J., Muir, G. D., Pataky, D. M., McBride, C. B., Petrusch, B., and Zwimpfer, T. J. (1994). Permissive and restrictive periods for brainstem-spinal regeneration in the chick. *Progress in Brain Research* 103, 243-62.

Swerlick, R. A., Yancey, K. B., and Lawley, T. J. (1988). A direct in vivo comparison of the inflammatory properties of human C5a and C5a des Arg in human skin. *Journal of Immunology* 140, 2376-81.

Taylor, J., Pesheva, P., and Schachner, M. (1993). Influence of janusin and tenascin on growth cone behavior in vitro. *Journal of Neuroscience Research* 35, 347-62.

Tello, F. (1911). La influencia del neurotropismo en la regeneracion de los centros nerviosos. *Trabajos del Lab de Invest. Biol.* 9, 124-159.

Tipping, P. G., Boyce, N. W., and Holdsworth, S. R. (1989). Relative contributions of chemo-attractant and terminal components of complement to anti-glomerular basement membrane (GBM) glomerulonephritis. *Clinical & Experimental Immunology* 78, 444-8.

Tolbert, D. L., and Der, T. (1987). Redirected growth of pyramidal tract axons following neonatal pyramidotomy in cats. *Journal of Comparative Neurology* 260, 299-311.

Tomlinson, S. (1993). Complement defense mechanisms. *Current Opinion in Immunology* 5, 83-9.

Touray, M. G., Seeley, D. C., Jr., and Miller, L. H. (1994). *Plasmodium gallinaceum*: differential lysis of two developmental stages of malaria sporozoites by the alternative pathway of complement. *Experimental Parasitology* 78, 294-301.

Trapp, B. D., Bernier, L., Andrews, S. B., and Colman, D. R. (1988). Cellular and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. *Journal of Neurochemistry* 51, 859-68.

Treherne, J. M., Woodward, S. K., Varga, Z. M., Ritchie, J. M., and Nicholls, J. G. (1992). Restoration of conduction and growth of axons through injured spinal cord of neonatal opossum in culture. *Proceedings of the National Academy of Sciences of the United States of America* 89, 431-4.

Unkeless, J. C., and Wright, S. D. (1988). Phagocytic Cells: Fc and Complement Receptors. In *Inflammation: Basic Principles and Clinical Correlates*, J. I. Gallin, I. M. Goldstein and R. Snyderman, eds. (New York: Raven Press Ltd.), pp. 343-363.

Vanderluit, J. L., Bourque, J. A., Peterson, A. C., and Tetzlaff, W. (2000). A model for focal demyelination of the spinal dorsal columns of transgenic MBP-LacZ mice by phototargeted ablation of oligodendrocytes. *Journal of Neuroscience Research In Press*.

Varga, Z. M., Bandtlow, C. E., Erulkar, S. D., Schwab, M. E., and Nicholls, J. G. (1995a). The critical period for repair of CNS of neonatal opossum (*Monodelphis domestica*) in culture: correlation with development of glial cells, myelin and growth-inhibitory molecules. *European Journal of Neuroscience* 7, 2119-29.

Varga, Z. M., Schwab, M. E., and Nicholls, J. G. (1995b). Myelin-associated neurite growth-inhibitory proteins and suppression of regeneration of immature mammalian spinal cord in culture. *Proceedings of the National Academy of Sciences of the United States of America* 92, 10959-63.

Veerhuis, R., van der Valk, P., Janssen, I., Zhan, S. S., Van Nostrand, W. E., and Eikelenboom, P. (1995). Complement activation in amyloid plaques in Alzheimer's disease brains does not proceed further than C3. *Virchows Archiv* 426, 603-10.

Verbrugh, H. A., Lee, D. A., Elliott, G. R., Keane, W. F., Hoidal, J. R., and Peterson, P. K. (1985). Opsonization of *Legionella pneumophila* in human serum: key roles for specific antibodies and the classical complement pathway. *Immunology* 54, 643-53.

von Zabern, I. (1988). Species-dependent incompatibilities. In *The Complement System*, K. Rother and G. O. Till, eds. (Berlin: Springer-Verlag), pp. 196-202.

Waehneltdt, T. W., Matthieu, J.-M., and Jeserich, G. (1986). Appearance of myelin proteins during vertebrate evolution. *Neurochem. Int.* 9, 463-474.

Waller, A. V. (1850). Experiments on the section of the glossopharyngeal and hypoglossal nerves in the frog and observation on the alterations produced thereby in the structure of their primitive fibers. *Phil. Trans. R. Soc. London (Biol.)* 140, 423.

Wanner, M., Lang, D. M., Bandtlow, C. E., Schwab, M. E., Bastmeyer, M., and Stuermer, C. A. (1995). Reevaluation of the growth-permissive substrate properties of goldfish optic nerve myelin and myelin proteins. *Journal of Neuroscience* 15, 7500-8.

Weibel, D., Cadelli, D., and Schwab, M. E. (1994). Regeneration of lesioned rat optic nerve is improved after neutralization of myelin associated growth inhibitors. *Brain Research* 642, 259-266.

Whaley, K. (1980). Biosynthesis of the complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. *Journal of Experimental Medicine* 151, 501-16.

Wright, S. D., and Griffin, F. M., Jr. (1985). Activation of phagocytic cells' C3 receptors for phagocytosis. *Journal of Leukocyte Biology* 38, 327-39.

Xu, X. M., Guenard, V., Kleitman, N., and Bunge, M. B. (1995). Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord. *Journal of Comparative Neurology* 351, 145-60.

Xu, X. M., and Martin, G. F. (1989). Developmental plasticity of the rubrospinal tract in the North American opossum. *Journal of Comparative Neurology* 279, 368-81.

Xu, X. M., and Martin, G. F. (1991). Evidence for new growth and regeneration of cut axons in developmental plasticity of the rubrospinal tract in the North American opossum. *Journal of Comparative Neurology* 313, 103-12.

Yajima, K., and Suzuki, K. (1979). Demyelination and remyelination in the rat central nervous system following ethidium bromide injection. *Laboratory Investigation* 41, 385-92.

Yam, P., Petz, L. D., Tourtellotte, W. W., and Ma, B. I. (1980). Measurement of complement components in cerebral spinal fluid by radioimmunoassay in patients with multiple sclerosis. *Clinical Immunology & Immunopathology* 17, 492-505.

Yao, J., Harvath, L., Gilbert, D. L., and Colton, C. A. (1990). Chemotaxis by a CNS macrophage, the microglia. *Journal of Neuroscience Research* 27, 36-42.

Yasuhara, O., Aimi, Y., McGeer, E. G., and McGeer, P. L. (1994). Expression of the complement membrane attack complex and its inhibitors in Pick disease brain. *Brain Research* 652, 346-9.

Zajicek, J. P., Wing, M., Scolding, N. J., and Compston, D. A. (1992). Interactions between oligodendrocytes and microglia. A major role for complement and tumour necrosis factor in oligodendrocyte adherence and killing. *Brain* 115, 1611-31.

Zeev-Brann, A. B., Lazarov-Spiegler, O., Brenner, T., and Schwartz, M. (1998). Differential effects of central and peripheral nerves on macrophages and microglia. *Glia* 23, 181-90.

Zhu, B., Wayne Moore, G. R., Zwimpfer, T. J., Kastrukoff, L. F., Dyer, J. K., Steeves, J. D., Paty, D. W., and Cynader, M. S. (1999). Axonal cytoskeleton changes in experimental optic neuritis. *Brain Research* 824, 204-217.