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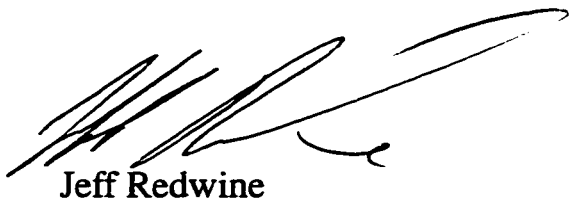

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ABSTRACT

Title of Dissertation: The *In Vivo* PDGF Response During Remyelination in Mouse Spinal Cord Following Murine Hepatitis Virus Strain A59-Induced Transient Demyelination

Jeff Redwine, PhD, 1998

Thesis directed by: Regina C. Armstrong, PhD, Associate Professor Departments of Anatomy and Cell Biology, Neuroscience, and Molecular and Cell Biology

Mechanisms involved in myelin repair (remyelination) are poorly understood. This project examined changes in oligodendrocyte function during remyelination in the adult central nervous system (CNS). Knowledge of how oligodendrocytes remyelinate adult CNS may lead to therapies for chronic human demyelinating diseases such as multiple sclerosis (MS). In MS, demyelination is followed by partial, but incomplete remyelination.

During remyelination, as happens during developmental myelination, oligodendrocytes may proliferate, migrate, and differentiate to repopulate and remyelinate demyelinated lesions. *In vitro* studies have shown that platelet-derived growth factor (PDGF) induces proliferation, migration,

and promotes the survival of oligodendrocyte progenitors. Basic fibroblast growth factor (bFGF) induces proliferation of oligodendrocyte lineage cells (OLCs). Therefore, these growth factors, particularly PDGF, may be involved in promoting remyelination *in vivo*.

I hypothesize that the re-expression or upregulation of the PDGF receptor in conjunction with locally available PDGF is associated with oligodendrocyte repopulation and remyelination of demyelinated lesions in the adult mouse CNS.

To test this hypothesis, a mouse model of acute demyelination followed by successful remyelination was used. Murine hepatitis virus strain A-59 (MHV-A59) causes an acute infection, followed by demyelination of the brain and spinal cord when intracranially injected into four-week-old mice. This demyelination is extensive enough to cause partial paresis or paralysis. Importantly, remyelination subsequently occurs throughout the CNS.

Following MHV-A59 induced demyelination, a stage of early remyelination was characterized by a motor test that documented motor recovery associated with histological evidence of remyelination. Multi-label immunofluorescence was used to characterize phenotypes of cells expressing PDGF, and receptors for PDGF and bFGF. To examine OLC proliferation *in vivo* related to expression of the PDGF receptor, *in situ*

hybridization in combination with bromodeoxyuridine (BrdU) pulse-labeling of proliferating cells was used.

It was found that OLCs express receptors for PDGF and bFGF. During remyelination, there are increases in the number of 1) cells expressing PDGF, 2) cells expressing PDGF receptor, and more importantly, 3) OLCs double labeled for PDGF receptor and BrdU incorporation. These findings demonstrate that PDGF is associated with OLC proliferation and repopulation of remyelinating lesions.

THE *IN VIVO* PDGF RESPONSE DURING REMYELINATION IN MOUSE SPINAL CORD FOLLOWING MURINE HEPATITIS VIRUS STRAIN A59-INDUCED TRANSIENT DEMYELINATION

by

Jeffrey Michael Redwine

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List of Abbreviations

bFGF.....basic fibroblast growth factor

BrdU.....bromodeoxyuridine

CNP.....2′,3′-cyclic nucleotide 3′ phosphodiesterase

CNS.....central nervous system

DIG.....digoxigenin

DMEM.....Dubelco's modified eagle medium

dpi.....days post injection

EAE.....experimental allergic encephalomyelitis

FBS.....fetal bovine serum

FGFR.....fibroblast growth factor receptor

GFAP.....glial fibrillary acidic protein

i.c. .....intracranial

IGF-1.....insulin-like growth factor-1

i.p. .....intraperitoneal

MHV-A59.....murine hepatitis virus strain A-59

MS.....multiple sclerosis

OLC.....oligodendrocyte lineage cell

PDGF.....platelet-derived growth factor

PDGFαR.....platelet-derived growth factor alpha recetor

PFU.....plaque forming unit

PLP.....proteolipid protein

PSF.....penicillin-streptomycin-fungizone

r.t. .....room temperature

SVZ.....sub-ventricular zone

wpi.....weeks post-injection

Chapter 1

Introduction and Background

This project was designed to examine mechanisms that may be involved in myelin repair of a transiently demyelinated adult mouse spinal cord. Knowledge of these mechanisms may lead to therapies that reduce the long term neurologic deficit of demyelinating diseases in animals and humans. Current knowledge of mechanisms involved in normal developmental myelination and *in vitro* oligodendrocyte function will be used to focus this research project on potential mechanisms that may be used in adult animal myelin regeneration.

During neonatal development, oligodendrocyte precursor/stem cells proliferate in germinal zones such as the subventricular forebrain or along the dorsoventral axis of the spinal cord near the central canal, migrate to future white matter areas, and differentiate into mature myelin-producing oligodendrocytes (Luskin and McDermott, 1994; Levison and Goldman, 1993; Pringle and Richardson, 1993). Germinal zones persist in the adult rodent central nervous system (CNS), and are thought to contain neuronal, astrocyte, and oligodendrocyte precursors (Gritti *et al.*, 1996; Young and Levison, 1996).

Mechanisms involved in remyelination may be similar to mechanisms involved in developmental myelination. In other words, in order to remyelinate, oligodendrocytes must repopulate demyelinated lesions by proliferating, migrating into demyelinated lesions, and then differentiating into myelin producing oligodendrocytes. In addition to the mature myelinating oligodendrocyte, adult oligodendrocyte progenitors have been identified *in vitro* and *in vivo*. This cell population persists in the adult CNS and is scattered throughout the white and gray matter. Oligodendrocyte lineage cells (OLCs) contributing to remyelination in adult CNS may be mature oligodendrocytes, or immature adult oligodendrocyte progenitors (discussed in a later section). This project focuses on the role of the adult oligodendrocyte progenitor during remyelination. A discussion of neonatal oligodendrocytes will be followed by a discussion of adult oligodendroctye progenitors and potential mechanisms regulating this phenotype during remyelination.

Neonatal Oligodendrocyte Progenitors

Since most myelination occurs postnatally, neonatal oligodendrocyte cultures contain a rich population of immature oligodendrocyte progenitors. Oligodendrocyte progenitors can be maintained in culture in

a defined medium (Eccleston and Silberberg, 1984; see Armstrong, 1998 for review) with growth factors added to maintain their immature, proliferative precursor phenotype. These progenitors can differentiate into mature, myelin protein-producing oligodendrocytes *in vitro* when growth factors are removed from the defined media (Raff *et al.*, 1983). Oligodendrocyte cultures provided investigators with the opportunity to 1) identify factors that regulate neonatal oligodendrocyte progenitor proliferation, migration, and differentiation *in vitro*, and 2) characterize an adult oligodendrocyte progenitor phenotype. The response of the adult oligodendrocyte progenitor during remyelination is examined in this project.

Neonatal oligodendrocyte progenitors were characterized by a small bipolar or tripolar morphology, and by expression of antigenic markers such as A2B5 (Raff *et al.*, 1983) and O4 (Sommer and Schachner, 1981; Schachner *et al.*, 1981) but not mature oligodendrocyte markers associated with myelin production, such as galactocerebroside (Raff *et al.*, 1978). Markers that have been used more recently to specifically label oligodendrocyte progenitors *in vitro* and *in vivo* are NG2 (Levine and Stallcup, 1987; Levine *et al.*, 1993; Nishiyama *et al.*, 1996; Stallcup and Beasley, 1987; Trapp *et al.*, 1997) and PDGFαR (Hart *et al.*, 1989;

Oumesmar *et al.*, 1997; Pringle *et al.*, 1992). NG2 and PDGFαR also specifically label an *adult* oligodendrocyte progenitor phenotype that will be discussed in a later section.

Neonatal Oligodendrocyte Progenitor Responses to Growth Factors *in vitro*

Certain molecules can regulate the *in vitro* proliferation, migration, differentiation, and survival of OLCs cultured from neonatal rodent CNS. Well-characterized molecules that affect OLCs are platelet derived growth factor AA homodimer (PDGF-AA), basic fibroblast growth factor (bFGF), insulin-like growth factor I (IGF-1), and transforming growth factor beta (TGFß). Most of the research examining growth factor effects on OLC's has been done *in vitro*. However there is more recent research *in vivo* that supports results obtained from *in vitro* experiments. A discussion of *in vitro* data regarding growth factors will be followed by a discussion of *in vivo* experiments examining growth factors and their receptors.

Growth factors induce neonatal oligodendrocyte progenitor proliferation in vitro

Neonatal oligodendrocyte progenitors proliferate in mixed glial cultures containing astrocytes (Noble and Murray, 1984). Neonatal oligodendrocyte progenitors also proliferated in cultures in response to

astrocyte conditioned media, and a factor secreted by astrocytes that induces proliferation was found to be PDGF-AA homodimer (Richardson *et al.*, 1988). PDGF-AA is a well-characterized mitogen for oligodendrocyte progenitors (Richardson *et al.*, 1988; Raff *et al.*, 1988; Noble *et al.*, 1988; Wolswijk *et al.*, 1991; Barres *et al.*, 1993; Engel and Wolswijk, 1996). Oligodendrocyte progenitors are known to express the receptor for PDGF-AA, PDGFα receptor (PDGFαR). Oligodendrocyte progenitors bind ^{125}I-labeled PDGF-AA (Hart *et al.*, 1989), and PDGFαR mRNA is found in cultured rat oligodendrocyte progenitors that bind GD3 or O4 antibodies (Ellison *et al.*, 1994). As will be discussed in later sections, PDGF-AA has multiple effects on OLCs that make it an excellent candidate for examining during remyelination, especially since very little is known about the role of PDGF during remyelination *in vivo*.

bFGF induces neonatal rat oligodendrocyte progenitors (McKinnon *et al.*, 1990) and mature oligodendrocytes (Fressinaud *et al.*, 1993) to proliferate, as indicated by increased BrdU incorporation when cultured with bFGF. bFGF has additional effects on OLCs in the presence of PDGF-AA. bFGF also potentiates the proliferative effects of PDGF on OLCs. Incubation of oligodendrocyte progenitors with bFGF and PDGF-AA combined increases the length of time that these cells proliferate,

causing them to grow as a self-renewing cell line (Bogler *et al.*, 1990; McKinnon *et al.*, 1990). This effect may be due in part to upregulation of PDGFαR mRNA (McKinnon *et al.*, 1990).

Other growth factors induce oligodendrocytes to proliferate in culture as well. Oligodendrocytes that do not express mature oligodendrocyte marker galactocerebroside incorporate ^{3}H-thymidine when cultured with IGF-1 (McMorris and Dubois-Dalq, 1988; McMorris *et al.*, 1990), and OLCs bind ^{125}I-labelled IGF-1 (McMorris *et al.*, 1986). In fibroblast cell lines, IGF-1 and PDGF act together to induce proliferation (Miura *et al.* 1994; Rubini *et al.*, 1994), however this interaction has not been observed in OLCs. The role of IGF-1 during remyelination has been more extensively examined than PDGF (discussed in later section).

OLC proliferation can also be inhibited by soluble growth factors. Transforming growth factor-beta (TGFß) inhibits proliferation of OLCs cultured in PDGF (McKinnon *et al.*, 1993b). TGFß may inhibit proliferation by increasing the rate of oligodendrocyte progenitor differentiation into mature oligodendrocytes (McKinnon *et al.*, 1993b). TGFß inhibition of PDGF-AA induced proliferation may involve other mechanisms. For example TGFß down-regulates PDGFαR mRNA in lung

fibroblasts (Bonner *et al.*, 1995). The effects of TGFß on OLCs *in vitro* and *in vivo* need to be further characterized.

Growth factors stimulate neonatal oligodendrocyte progenitor migration in vitro.

Migration of neonatal OLCs is regulated by extracellular soluble molecules (Armstrong *et al.*, 1990), and by substrate interactions (Fok-Seang *et al.*, 1995; Bartch *et al.*, 1992). OLC substrate interactions are beyond the focus of this project. The most potent soluble chemoattractant known for OLCs is PDGF-AA. Rat neonatal oligodendrocyte progenitors migrate towards an increased concentration gradient of soluble PDGF-AA in a microchemotaxis chamber, and to a lesser extent bFGF, but not to IGF-1, or TGFß (Armstrong, *et al.*, 1990a). Also, cultured rat neonatal oligodendrocyte progenitors are migratory in the presence of PDGF or PDGF and bFGF combined, but not in the presence of bFGF alone when observed with time-lapsed microcinematography (McKinnon *et al.*, 1993).

Growth factors modulate OLC differentiation in vitro.

IGF-1 increases the number of oligodendrocyte progenitors that express myelin specific markers in culture, and is therefore thought to be a differentiation factor for OLCs. The percentage of oligodendrocyte

precursors cultured from neonatal rodent that differentiate into mature oligodendrocytes increases in the presence of IGF-1 (McMorris and Dubois-Dalcq, 1988, McMorris *et al.*, 1990) or triiodothyronine (T3) (Barres *et al.*, 1994; Ahlgren *et al.*, 1997; Baas *et al.*, 1997; Shi *et al.*, 1998). Therefore, IGF-1 and T3 are thought to be differentiation factors.

Incubation of cultured mature oligodendrocytes in bFGF has a reverse effect, characterized by reducing the percentage of oligodendrocytes that express mature oligodendrocyte markers (Grinspan *et al.*, 1993; Bansal and Pfeiffer, 1997).

Growth factors promote oligodendrocyte survival <u>in vitro</u>.

Oligodendrocyte progenitor cells die in culture in the absence of growth factors, often displaying a morphology resembling apoptotic death (shrunken, condensed nuclei, compact chromatin; Barres *et al.*, 1992). However, the number of OLCs cultured from neonatal rat optic nerve that survive is increased when incubated with PDGF-AA or IGF-1 (Barres *et al.*, 1992). Other growth factors, or combinations of growth factors, may also increase the survival rate of OLCs in culture. Examples of such factors are neurotrophin-3, ciliary neurotrophic factor, leukemia inhibitory factor, and interleukin-6 (Barres *et al.*, 1993).

Data from in vitro studies have increased knowledge of oligodendrocyte function in vivo.

Some of the growth factors that affect OLC function *in vitro* may also affect OLC function *in vivo.* For PDGF, results from studies showed the presence of the ligand and the receptor *in vivo,* as well as changes in the oligodendrocyte progenitor population when PDGF-AA levels are increased. For IGF-1, results from studies have shown the presence of the ligand *in vivo*, as well as changes in CNS myelination when IGF-1 levels were altered.

Oligodendrocyte progenitors in neonatal rat brain and optic nerve express PDGFαR mRNA transcripts (Mudhar *et al.* 1993; Ellison and de Vellis, 1994). PDGF-A is also present *in vivo.* PDGF-A immunoreactivity was detected in astrocytes within the optic nerve (Mudhar *et al.*, 1993), and motor neurons express PDGF-A chain mRNA (Yeh *et al.*, 1991). Neonatal oligodendrocyte progenitors likely respond to PDGF-AA *in vivo*, since a local increase in PDGF-AA levels in the rat optic nerve produced by transformed fibroblasts injected into the optic nerve causes an increase in the number of oligodendrocytes identified at a later time point (Barres *et al.*, 1992). This "response" to increased PDGF-AA levels may be

increased proliferation and/or survival.

There is also evidence that IGF-1 is involved in normal oligodendrocyte development. Oligodendrocytes expressing O4 antigen isolated from neonatal rats also express IGF-1 mRNA (Shinar and McMorris, 1995). IGF-1 transgene models provide strong evidence that IGF-1 levels enhance oligodendrocyte's ability to myelinate the CNS. Over-expression of IGF-1 results in increased myelin content of the CNS, increased thickness of myelin sheaths, and an increased number of myelinated axons (Ye *et al.*, 1995; Carson *et al.*, 1993). Conversely, the few mice that survive an IGF-1 gene disruption have underdeveloped white matter in brainstem and spinal cord (Liu *et al.*, 1993). Reduction of IGF-1 levels in IGF-1 knockout mice results in decreased myelination of the CNS (Ye *et al.*, 1995; Beck *et al.*, 1995). However, one study suggests that reduced myelin seen in brains of adult IGF-1 null mice is related to reduced axon size and/or reduced number of axons (Cheng *et al.*, 1998). Oligodendrocyte development *in vivo* may involve factors in addition to IGF-1, such as axonal contact, and activation of the IGF-1 receptor via redundant or compensatory molecules such as IGF-II or insulin.

Characterization of an adult oligodendrocyte progenitor phenotype

Not long after neonatal oligodendrocyte progenitor culture techniques were refined, oligodendrocyte progenitors cultured from *adult* rodent optic nerve were identified (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). These original studies identified oligodendrocyte progenitors as cells that express oligodendrocyte progenitor markers, but do not express mature oligodendrocyte markers, such as galactocerebroside, or astrocyte marker GFAP. However results from a recent study showed adult oligodendrocyte progenitors "acutely" express mature oligodendrocyte marker galactocerebroside in culture immediately following isolation and plating (at an early time point not previously examined), but then lose this marker at later time points (Shi *et al.*, 1998). The existence of this progenitor phenotype (adult oligodendrocyte progenitor expressing galactocerebroside) remains to be confirmed *in vivo*. Adult oligodendrocyte progenitors were induced to differentiate into mature oligodendrocytes with elaborate processes when animal serum was from the media (Shi *et al.*, 1998). Differentiation of neonatal oligodendrocyte progenitors also occurs when animal serum is removed from the media. (ffrench-Constant and Raff, 1986; Shi *et al.*, 1998). In addition, OLCs that express oligodendrocyte progenitor

markers, but not mature oligodendrocyte markers have been cultured from human CNS (Armstrong *et al.*, 1992; Gogate *et al.*, 1994; Prabhaker *et al.*, 1995; Scolding *et al.*, 1995).

In addition to oligodendrocyte progenitors cultured from adult rodent and human CNS, adult oligodendrocyte progenitors have been identified *in situ* in rodent CNS (Levine *et al.*, 1993; Reynolds and Hardy, 1997) and in human brain (Armstrong *et al.*, 1992). Additional studies have confirmed the existence of an adult oligodendrocyte progenitor cell population *in vivo*. Based on these studies, the most reliable and best characterized markers for rodent adult oligodendrocyte progenitors both *in vitro* and *in vivo* are NG2 and PDGFαR (Levine *et al.*, 1993; Nishiyama *et al.*, 1996, 1997; Redwine *et al.*, 1997; Reynolds and Hardy, 1997; Trapp *et al.*, 1997). Progressive stages of differentiation from NG2+ oligodendrocyte progenitors to oligodendrocytes have been demonstrated *in vitro* and *in vivo* (Levine *et al.*, 1993; Reynolds and Hardy, 1997; Trapp *et al.*, 1997).

Adult oligodendrocyte progenitors proliferate in response to growth factors.

Despite the existence of an oligodendrocyte progenitor in the

neonatal and adult CNS, adult oligodendrocyte progenitors differ in some ways from neonatal oligodendrocyte progenitors. Although adult oligodendrocyte progenitors proliferate *in vitro* (Wolswijk and Noble, 1989) and *in vivo* (Levine *et al.*, 1993), adult oligodendrocyte progenitors have a longer cell cycle period, are less motile, and have more long, thin processes than are found on neonatal oligodendrocyte progenitors (Wolswijk and Noble, 1989).

However, adult oligodendrocyte progenitors cultured from rat optic nerve or spinal cord with PDGF-AA and bFGF combined in the media display proliferative, motility, and morphological characteristics similar to neonatal oligodendrocyte progenitors (Wolswijk and Noble, 1992; Engel and Wolswijk, 1996). An increased proliferation of adult oligodendrocyte progenitors was also reported in response to the combined presence of PDGF-AA and glial growth factor-2 (GGF2) in cultures (Shi *et al.*, 1998).

The response of adult oligodendrocyte progenitors cultured from human CNS to soluble factors has also been tested. IGF-1 induces differentiation of human adult oligodendrocyte progenitors. bFGF increases the percent of OLCs that have a progenitor phenotype (Armstrong *et al.*, 1992) and induces process formation in OLCs (Gogate *et al.*, 1994; Oh and Yong, 1996; Oh *et al.*, 1997). Although human adult

oligodendrocyte progenitors do not proliferate in response to growth factors (Armstrong *et al.*, 1992; Gogate *et al.*, 1994; Scolding *et al.*, 1995), they do proliferate when cultured on an astrocyte monolayer (Scolding *et al.*, 1995). Conditions for primary human glial culture may still require optimizing.

The response of rodent adult oligodendrocyte progenitors to combined growth factors such as PDGF and bFGF is important, because during remyelination in the adult CNS, adult oligodendrocyte progenitors may be most efficient at repopulating demyelinated lesions if they respond to both PDGF-AA and bFGF, *i.e.*, if they express receptors for both growth factors. Prior to this project, the receptors for these growth factors on *in vivo* oligodendrocyte progenitors was not documented. Data regarding expression of these receptors on adult oligodendrocyte progenitors in normal and remyelinating tissue are presented in chapters 3 and 4.

Models of demyelination and remyelination

Several conditions in humans and animals cause demyelination of axons in the adult CNS, and thus require regeneration of OLCs for remyelination. For example, in human multiple sclerosis, remyelination

has been documented in lesions, however, the extent appears insufficient or only transient, possibly due to recurring disease activity (Raine and Wu, 1993; Prineas *et al.*, 1993; Ozawa *et al.*, 1994). Therefore, chronicly, insufficient repair of demyelinated lesions results in sensory and motor dysfunction (reviewed in ffrench-Constant, 1994).

A similar pathological process in the CNS is observed in an animal model of MS, experimental autoimmune encephalomyelitis (EAE). EAE can be induced in rodents by injecting myelin components, such as myelin basic protein and/or galactocerebroside (Raine *et al.*, 1988), or by passive transfer of activated T lymphocytes (Lannes-Vieira *et al.*, 1994). Clinical paresis or paralysis of one or more limbs occurs in a chronic, relapsing and remitting pattern, that is similar to the clinical pattern seen in human MS. A similar pattern of demyelination with relapsing, remitting paresis can be induced by inoculation with Theiler's murine encephalitis virus (TMEV) (reviewed in Miller *et al.*, 1995).

Contrary to EAE and TMEV, there are animal models of acute, or transient adult CNS demyelination followed by remyelination. Transient demyelination, followed by remyelination can be induced by injecting chemicals such as lysolecithin into the brain or spinal cord. Some results from studies show chemically inducing demyelination, and either observe

the subsequent endogenous myelin repair (Komoly *et al.* 1992), or increase the rate of subsequent myelin repair by transplanting exogenous oligodendrocyte precursors into the lesioned area (Gout *et al.*, 1988; Vignais *et al.*, 1993; Trotter *et al.*, 1993). In this project we used a mouse coronavirus model that is characterized by demyelination of the CNS followed by successful remyelination (Lavi *et al.*, 1984a, 1984b, Kristensson *et al.*, 1986, Jordan *et al.*, 1989a, 1989b, Godfraind *et al.*, 1989, Armstrong *et al.*, 1990b). This model is excellent for examining remyelination, because 1) focal demyelinated lesions occur throughout the CNS in a pattern similar to that seen in human MS, 2) the demyelination is severe enough to cause motor dysfunction, and 3) the demyelination is successfully repaired. Also, there is a complex cellular response within lesions in this model that resembles the cellular complexity associated with MS plaques. A more complete description of this model is given in Chapter 3.

Cellular and molecular mechanisms of remyelination

In order to remyelinate, oligodendrocytes must initially proliferate and/or migrate to repopulate demyelinated lesions. This project examined growth factor responses associated with OLC proliferation during

remyelination. OLCs capable of repopulating a demyelinated lesion may be mature myelinating oligodendrocytes and/or oligodendrocyte progenitors. It is known that OLCs proliferate *in vivo* in response to demyelinating damage, however it is not known what mechanisms induce endogenous repopulation of demyelinated lesions. Although this project examines the proliferative response during remyelination, other studies have examined migration into demyelinated lesions. Transplanted oligodendrocyte precursors migrate from the injection site, produce myelin, and increase the rate that myelin reappears in chemically-induced demyelinated lesions (Gout *et al.*, 1988; Trotter *et al.*, 1993). Migration of endogenous OLCs into demyelinated lesions may also occur from the border of lesions (Franklin *et al.*, 1997).

Growth factors may regulate OLC ability to remyelinate. The role of IGF-1 has been examined during remyelination in a model of transient demyelination induced by lysolecithin. During remyelination following chemically induced demyelination in mouse brain, astrocytes express IGF-1, and O4+ oligodendrocytes express the IGF-1 receptor (Komoly *et al.*, 1992). Also, peripheral administration of IGF-1 to rats has beneficial effects in three different models of EAE. IGF-1 therapy reduces the severity of clinical deficits (Liu *et al.*, 1995; Yao *et al.*, 1995), reduces the

extent of demyelination or lesion areas (Yao *et al.*, 1995; Liu *et al.*, 1997; Li *et al.*, 1998), and increases mRNA levels for myelin specific genes such as myelin basic protein compared to non-treated controls (Yao *et al.*, 1995). *In vitro*, incubation of IGF-1 with OLCs cultured from demyelinated spinal cord (induced by MHV-A59) increases the ratio of mature, galactocerebroside positive oligodendrocytes to other cell types (type-2 astrocyte) (Armstrong *et al.*, 1990b). Thus, IGF-1 appears to play a role in myelin repair of the adult CNS.

Oligodendrocyte lineage cells proliferate in response to demyelination.

Mature oligodendrocytes incorporate ^{3}H-thymidine (Herndon *et al.*, 1977; Arenella and Herndon, 1984; Prayoonwiwat and Rodriguez, 1993) *in vivo* in response to acute demyelinating damage, suggesting that these cells proliferate. However, results from these studies showed proliferating mature oligodendrocytes only when there was a significant amount of time (such as days or weeks) between the proliferation marker injection and animal sacrifice. It is not clear, therefore, if the proliferating cells were progenitors or mature oligodendrocytes at the time the ^{3}H-thymidine was incorporated into the cell nucleus. This present study complements previous studies by identifying proliferating cells *in vivo* following a short

time period that does not allow time for phenotype changes between proliferation marker injection and animal sacrifice. Other studies have identifed adult oligodendrocyte progenitors that proliferated in response to demyelinating damage (Gensert and Goldman, 1997; Keirstead *et al.*, 1998). However, these studies did not examine mechanisms associated with OLC proliferation, such as growth factor or growth factor receptor expression. The present study complements and extends previous studies by examining mechanisms such as growth factor and growth factor receptor expression associated with proliferation *in vivo.*

During remyelination in the MHV-A59 model, oligodendrocyte progenitors proliferate *in vitro* (Armstrong *et al.*, 1990b). *In vivo*, oligodendrocyte progenitors defined by binding O4 antibody, but not myelin specific CNP antibody (O4+/CNP-), are found within white matter lesions, and some of them incorporate ^{3}H-thymidine (Godfraind *et al.*, 1989), which is evidence that adult oligodendrocytes proliferate in response to demyelination.

Growth factors may promote OLC proliferation in response to demyelination.

No previous studies have examined proliferation associated with expression of growth factors, such as PDGF-AA or bFGF, or growth

factor receptors, such as PDGFαR or FGFRs, during remyelination. This study examined the PDGF pathway during remyelination in the MHV-A59 transient demyelination model. PDGF plays multiple roles during development, such as inducing OLC proliferation, migration, and promoting OLC survival. Since myelin repair in the adult CNS is hypothesized to require OLC proliferation, migration, and survival, PDGF may play a role in adult CNS remyelination. This study extends *in vitro* studies that implicate PDGF involvement in promoting myelin repair by examining remyelination *in vivo* in the MHV-A59 transient demyelination model. First, a time point that corresponds to early remyelination was characterized by examining histologic evidence of remyelination, and motor function improvement following demyelination (Chapter 3). Second, adult oligodendrocyte progenitors were identified in normal and remyelinating spinal cord, and expression of receptors for PDGF-AA and bFGF were identified on this cell population *in vivo*. This experiment is significant since *in vitro* data suggest adult oligodendrocyte progenitors would be more able to repopulate demyelinated lesions if they respond to both growth factors (Chapter 4). Third, expression of PDGF-A and the PDGFαR was examined during early remyelination, along with the association of PDGFαR to adult oligodendrocyte progenitors and to

proliferating cells (Chapter 5).

By examining a model of functionally complete adult remyelination, mechanisms of remyelination in adult animals may be better understood. Ultimately, therapies may be developed to enhance oligodendrocyte functions critical for remyelination in chronic demyelinating diseases such as MS.

Chapter 2

Experimental Design And Methods

To examine cellular and growth factor responses occurring during remyelination in the mouse, a coronavirus (murine hepatitis virus strain A59) was used to induce transient demyelination. Mice were sacrificed at one time point during the early stages of spontaneous remyelination that follows the virally-induced demyelination. Mouse spinal cord tissue was then examined for cellular and growth factor responses, as well as cell proliferation associated with remyelination.

Animals

Twenty one day old C57Bl/6 female mice were purchased from Jackson Laboratories or from Charles River Laboratories. Mice were housed in an isolated animal room 5 mice per cage, with free access to food and water. To reduce unintentional contamination by the virus used in this model, each cage was covered with a filter top, and placed in a filtered negative air flow rack. Mice were housed for 7 days prior to experiments. Mice weighed approximately 10-16 grams on the day of viral injection (28 days old). From 5-14 days after the viral injection, apple slices were placed in the bottom of cages to reduce potential dehydration.

MHV-A59 stock

Murine hepatitis virus strain A59 was kindly provided by Kathryn V. Holmes (Dept. of Microbiology, University of Colorado Health Sciences Center). The virus stock was collected on the fifth passage on 17-Cl-1 mouse fibroblasts, and a plaque assay was used to determine virus titer. The virus stock was diluted in sterile PBS to 1000 plaque-forming units (PFU) per 10 µl injection volume. The diluted virus was back titrated by plaque assay to verify the titer.

Plaque assay

A plaque assay was used to verify virus titer. Mouse fibroblasts (L2 from ATCC, Fairfax, VA; or 17-Cl-1 provided by K. Holmes) were grown in Dulbecco's-modifed Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin-fungizone mixture (PSF) (Life Technologies, Gaithersburg, MD).

Three days prior to the plaque assay, the cells were split 1:10 into 6-well plates, so they would grow to approximately 90% confluence on the day of virus titration.

Ten-fold dilutions of MHV-A59 (either the stock or the diluted vial used for injections) were made in DMEM/10% FBS. The supernatant in the 6 well plates was then aspirated and the fibroblasts were rinsed with

PBS. One hundred microliters (µl) of each dilution was gently added to cells (3 wells for each dilution for triplicate data points). The cells were incubated for 1 hr at 37°C, and the plates were manually rocked back and forth every 15 min. The cells were then overlaid with approximately 3 ml of agar solution containing 0.95% Noble agar (Sigma, St. Louis, MO) in minimum essential medium (MEM) (Life Technologies), 5% FBS, and 0.5% PSF. The agar solution was allowed to harden for approximately 30 min. at room temperature, and then cells were returned to the 37°C incubator where plaques were allowed to develop for 36-48 hours.

To detect plaques, a neutral red solution, containing 0.02% neutral red (Sigma) and 0.95% Noble Agar in MEM, was added to each well. The cells were incubated at 37°C for an additional 2-4 hrs. Round, unstained areas within the cell monolayer that indicate areas of dead cells (*ie.*, plaques) were counted for each serial dilution. A concentration of PFUs/ml virus stock was then calculated.

MHV-A59 injection

Twenty-eight-day-old mice were anesthetized with methoxyflurane inhalant anesthetic. The right side of the head was cleaned with alcohol, and a 10 µl volume was injected intracranially (i.c.) midway between the

right ear and right eye with a 25 gauge needle on a syringe. A STEPPER repetitive pipette (Tridak) was used to ensure accurate and consistent injection volumes. This injection method replicates the injection method used previously in this model (Armstrong *et al.*, 1990), and is consistent with other models of virally-induced CNS demyelination (Dal Canto *et al.*, 1995).

Motor performance test

Mice were placed on a hand-held cage top (cat# WBL7115; $7^1/4"$ x $11^1/2"$ x $5^1/2"$, Allentown Caging, Allentown, PA). The cage top was then turned upside down and held 18-24 inches above a table top. The cage top was taped around the edges to prevent mice from climbing to the other side, and was held so that the bars mice were holding onto were horizontal. The length of time the mice were able to hold onto the cage top while upside-down was recorded (as "hang time"), for a maximum of 60 seconds. A plastic bag stuffed with paper towels was placed on the table top and used to cushion any falls from the cage top. If mice held on for 60 seconds, they were removed from the cage top and allowed to rest for approximately 5 seconds between trials. For each test day, a mean of three trials was used as the recorded hang time. Hang times were recorded 0, 7,

10, 14, 17, 22, and 29 days post-injection (dpi). Some mice were also monitored 38 and 45 dpi. In pilot trials, mice were able to hang upside-down for as long as tested, up to six minutes (unpublished observations). The 60 second maximum was found to be sensitive enough to detect deficits and subsequent improvements in performance. In other pilot trials, a metal grid was used instead of the cage tops. The metal grid was easier for the mice to hang onto, since they could lodge their hind paws and/or part of their hind legs in the corner of the grids to prevent falls. This made the metal grid less sensitive and so was not selected for the motor performance test.

Clinical score scale

In addition to the motor performance test, mice were given a clinical score based on observed paresis or paralysis of limbs, so that 0 = no observable paresis/paralysis, 1-5 = observed paresis or paralysis in 1-5 limbs, respectively (such as limp tail, abnormal limb contracture or extension, dragging of limb, abnormal lateral movement of limb), and 6= morbidity (adopted from Lannes-Vieira, Gehrmann, Kreitzberg, & Werkele, 1994; Lavi *et al.*, 1994; Miller *et al.*, 1992). Clinical scores were recorded on the same days that hang times were monitored (0, 7, 10,

14, 17, 22, and 28 dpi).

Mouse weights

Mouse weights were recorded on the same days the hang times and clinical scores were monitored.

Tissue preparation

At 4 weeks post-injection (wpi), mice were perfused with 4% paraformaldehyde. The head was cut off, and skin and muscle were removed to expose the vertebrae. The vertabrae were carefully removed with scissors and forceps so that the dorsal spinal cord was exposed. The spinal cords were separated into cervical, thoracic, and lumbar sections with a straight razor, and then carefully removed from remaining vertabrae and meninges with forceps. These spinal cord segments were post-fixed by overnight immersion in 4% paraformaldehyde at 4°C. Some spinal cord segments were stained with osmium tetroxide, embedded in epoxy resin, cut as 1μm sections, and stained with toluidine blue to verify the extent of demyelination and remyelination (2 wpi, 4 wpi, 24 wpi). These sections were photographed on Technical Pan film (Kodak, Rochester, NY). For immunostaining and *in situ* hybridization, spinal cord

segments were cryoprotected by overnight infiltration with 30% sucrose, embedded in Tissue Tek O.C.T. compound, and stored at -80°C. Spinal cord sections were cut at 15 µm, thaw-mounted onto gelatin coated slides and stored for immunohistochemistry at -20°C, or mounted onto Superfrost Plus slides (Fisher, Pittsburg, PA) and stored at -80°C until the *in situ* hybridization technique was performed.

Immunofluorescence

Transverse thaw mounted sections 15 µm thick were rehydrated in PBS 3 changes for 3 minutes each (3 x 3 min.). The tissue was then treated with one of several pretreatments, depending upon the primary antibodies used (see Table 1). Sections were then incubated in a solution containing 25% normal goat serum, 1% BSA, 0.4% Triton-X 100, and 100 mM L-lysine prior to incubation with the primary antibody.

Primary antibodies used are listed in Table 1. The source, dilutions used, special pretreatments performed, and references for each antibody are also listed in Table 1. All priary amntibodies were diluted in PBS/3% BSA (unless otherwise stated) and incubated on tissue overnight at 4 °C.

PDGFαR expression was detected with a rabbit polyclonal IgG antibody (diluted 1:1000; generous gift of Carl-Henrik Heldin; Hermanson *et al.*, 1992). PDGF ligand was detected with a rabbit IgG polyclonal

antibody (diluted 1:200; Upstate Biotechnology cat. #06-130, Lake Placid, NY) raised against PDGF-A which recognizes PDGF-AA homodimer and PDGF-AB heterodimer with less than 10% crossreactivity with PDGF-BB.

Oligodendrocyte progenitors were identified using rabbit polyclonal antibodies against NG2 chondroitin sulfate proteoglycan (generous gifts from Joel Levine and William Stallcup; Levine *et al.*, 1993; Nishiyama *et al.*, 1996; 1997; Reynolds and Hardy, 1997) diluted 1:500. Microglia were identified by immunolabeling for Mac-1 with a rat monoclonal IgG2b antibody (Boehringer Mannheim, Indianapolis, IN; Ohno *et al.*, 1993), which was detected with tyramide amplification using the Renaissance indirect blue kit (New England Nuclear, Boston, MA). Mature oligodendrocytes were identified by expression of 2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNP), which was detected with a mouse monoclonal IgG1 antibody clone Mab-46-1 (a generous gift from F. Authur McMorris; Raible and McMorris, 1989). Oligodendrocytes and myelin were also identified with a rat monoclonal IgG antibody clone AB3 that recognizes two isoforms of proteolipid protein (PLP) (Immunodiagnostics, Bedford, MA). B Lymphocytes were identified with a monoclonal antibody clone RA3-6B2 that recognizes the CD45 receptor (Pharmingen, San Diego, CA). Bromo-deoxy uridine was identified with a monoclonal antibody

Antigen or Clone	Antibody	Animal Source/ Isotype	Non-standard pre-treatment	Dilu-tion	Cell type identified	Source	Reference
Mab-46-1	anti-CNP	mouse monoclonal IgG1		1:500	oligoden-drocyte	G. Reiser	Reiser et al., 1994
clone BMG 6H8	anti-BrdU F(ab)2 frag peroxidase conjugated	mouse monoclonal	2N HCl 30 min	1:15	proliferat-ing cells	Boehringer Mannheim	
clone RA3-6B2	anti-mouse CD45R /B220				B lymphocyte	Pharmingen	Hardy et al. (1991)
clone AB3	anti-rat PLP /DM20	rat monoclonal IgG	microwave 50% power 45 sec/ 10% triton 30 min.	1:50	oligoden-drocyte (mature)	Immuno-diagnostics	Armstrong et al., 1995
chondroitin sulfate proteo-glycan	anti-NG2	rabbit polyclonal	10 ug/ml proteinase K in 20 mM Tris pH 7.5 20 min 37 °C	1:500	oligoden-drocyte progenitor	J. Levine	Levine & Card. 1987 Nishiyama et al., 1997
Clone M1/70 (9)	anti-Mac-1	rat monoclonal IgG2b		1:200 + tyramide amp.	microglia/ macrophage	Boehringer Mannheim	Ohno et al., 1993
clone G-A-5	anti-GFAP	mouse monoclonal IgG1		1:20	astrocyte	Boehringer Mannheim	
GFAP	anti-GFAP	rabbit polyclonal		1:200	astrocyte	Sigma	
PDGF alpha receptor	anti-PDGF alpha receptor	rabbit polyclonal IgG		1:1000		C.H. Heldin	Herman-son et al., 1992
clone VBS-1	anti-FGFR	mouse monoclonal IgM	100mM Tris pH 9-10 30 min 37 °C	1:10		Chemicon	Venkates-waran et al., 1992
PDGF-A	anti-PDGF-A	rabbit polyclonal IgG		1:200		Upstate Biotechnol. (#06-130)	

clone BMG-6H8 conjugated to peroxidase (Boehringer Mannheim). Astrocyes were identified with antibodies against glial fibrillary acidic protein (GFAP). Either a rabbit polyclonal IgG anti-GFAP antibody (diluted 1:200; Sigma, St. Louis, MO) or a mouse monoclonal IgG1 anti-GFAP antibody (diluted 1:20; Boehringer Mannheim) was used, depending upon the requirements of the multi-labeling protocol.

For detection of FGFR expression (in combination with PDGFαR, NG2, or GFAP), tissue was pretreated with 100 mM Tris base, pH 9-10, for 30 min at 37°C, prior to incubation with mouse monoclonal IgM anti-FGFR (diluted 1:10; Clone VBS-1 from Chemicon, Temecula, CA, and Biogenesis, Sandown, NH), which recognizes mouse FGFR1, but may also recognize FGFR2 to a lesser extent, based on western blot analysis and competitive binding of bFGF (Venkateswaran *et al.*, 1992).

Following the primary antibody incubation, tissue was rinsed in PBS (3 x 3 min.), and blocked with PBS/ 3% BSA/ 5% animal serum (either goat or donkey serum) for 20 min. at room temperature.

All secondary antibodies were affinity purified $F(ab')_2$ fragments previously tested for minimal cross-reactivity to serum proteins from the other species used in each protocol (Jackson Immunoresearch, West Grove, PA). The secondary antibodies were conjugated to one of several

fluorochromes: FITC (fluorescein isothiocyanate), AMCA (7-amino-4-methylcoumarin-3-acetic acid), or Cy3 (indocarbocyanine). For each of the multi-labeling protocols used, combinations of immunostains were tested for cross-reactivity by omitting each primary antibody in series. In each case the protocols were optimized so that signal was not observed when the primary antibody was omitted. Sections were mounted with Vectashield (Vector Labs, Burlingame, CA).

Secondary antibodies were incubated on tissue for 1 hr. at room temperature. Tissue was then rinsed in PBS (3 x 3 min.), and mounted with Vectashield (Vector Labs, Burlingame, CA).

Immunofluorescence Imaging

Immunoreactivity was detected using a Zeiss Photoscope III epifluorescent microscope. Images obtained with a 6.3x or 25x objective were photographed with 400 ASA TMAX or Ektachrome film and then the negatives or slides were either printed directly from film or digitized at 400 dpi and imported into Adobe Photoshop. High resolution images, obtained with the 63x objective, were acquired and restored using the CELLscan image analysis system (Scanalytics Inc., Falls Church, VA) with a Photometrics CCD camera. Restoration with exhaustive photon reassignment produced images with a resolution of 0.25 μm in the x-y

dimensions and 0.5 μm in the z-dimension. Exhaustive photon reassignment uses a deconvolution algorithm (Carrington *et al.*, 1990) to remove out-of-focus fluorescence flaring that results with high magnification objectives. To perform exhaustive photon reassignment, standards for flaring or "point spread functions" were prepared using subresolution (0.2 μm) polystyrene microspheres (PS-Speck kit, Molecular Probes, Eugene, OR) that fluoresced in each of the imaging channels (red, green, blue) used for multi-label detection. The excitation and emission filter sets did not result in bleed-through of fluorescent signal from one fluorochrome channel to another. Colocalization analysis was performed by merging image sets using the CELLscan software. Colocalization was also tested by imaging the specimens with a triple band pass filter (Chroma Technologies, Brattleboro, VT) to simultaneously detect rhodamine or Cy3, fluorescein or Cy2, and AMCA. Three-dimensional reconstructions from z-series stacks of 25-40 optical planes collected at 0.25 μm apart were performed using Metamorph software (Universal Imaging, West Chester, PA).

In situ hybridization and BrdU labeling

The cDNA template for proteolipid protein (PLP) ribonucleotide

probe (kindly provided by Lynn Hudson) is complementary to an approximately 980 base mRNA fragment encoding a portion of the 5′ untranslated region and the entire coding region of mouse PLP mRNA (Hudson *et al.*, 1987). The cDNA template for PDGFαR (kindly provided by Bill Richardson) used is complementary to an approximately 1.5 kb mRNA fragment encoding a portion of the extracellular domain of the rat PDGFαR (Lee *et al.*, 1990, Mudhar *et al.*, 1993). This PDGFαR rat riboprobe has previously been shown to hybridize specifically to PDGFαR mRNA in mouse CNS (Vignais *et al.*, 1995). The cDNA template for PDGF-A (kindly provided by Mark Mercola) is a 0.9 kb fragment complementary to mouse PDGF-A chain mRNA.

A standard transformation protocol was used to amplify circular plasmids (containing PDGFαR or PDGF-A cDNA sequences) in DH5α competent bacteria (Life Technologies). Small molecular weight, circular DNA was then purified from bacteria with a DNA purification kit (mini-prep kit from Ambion, Austin, TX for PDGFαR template; maxi-prep kit from Promega for PDGF-A template).

The constructs were linearized with appropriate restriction enzymes, and purified with a DNA purification kit (Ambion). For PDGFαR, sense and antisense templates were prepared. For PDGF-A, only the template

used to transcribe an antisense RNA probe was made. RNA probes were transcribed from the linearized templates with an *in vitro* transcription kit (Ambion) to incorporate digoxigenin-UTP (Boehringer Mannheim). To verify DIG-incorporation and to check the specific activity of the DIG labeled RNA probes, a dot blot was performed (DIG wash and block buffer set, Boehringer Mannheim). Serial 10-fold dilutions were made of the labeled RNA. One µl of each dilution was cross-linked with ultra-violet light (UV Stratalinker, Strategene, La Jolla, CA) onto a positively charged nylon membrane (Boehringer Mannheim). The membrane was placed in 1X wash buffer for 1 min. followed by 1X block buffer for 5 min. The membrane was then incubated in anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:5000 in block buffer for 1 hr. The membrane was then washed in wash buffer, incubated in 1X detection buffer, and incubated in a color substrate solution containing 1X detection buffer, nitro blue tetrazolium chloride (NBT 450 µg/ml, Boehringer Mannheim), and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP 175 µg/ml, Boehringer Mannheim) for approximately 1 hr. To estimate concentration of labeled probes, the color intensity of dilutions from labeled probes was compared to matching dilutions of control DIG-labeled RNA provided with the kit.

The *in situ* hybridization method used for this project was designed

with the help of Regina Armstrong and Donna Messersmith, and was modified from previously published protocols (Armstrong *et al.*, 1995; Gao & Hollyfield, 1995; Kim *et al.*, 1997). Slides were rehydrated in PBS, fixed in 4% paraformaldehyde, and incubated in 10 μg/ml proteinase K (Sigma) for 20 min. at 37°C. The slides were acetylated, and pre-hybridized with mRNA hybridization buffer (DAKO, Carpinteria, CA) for 3-4 hours at room temperature (r.t.). DIG-labeled ribonucleotide probes were diluted to 400 ng/ml (for PLP and PDGF-A) or 200 ng/ml (for PDGFαR) in hybridization buffer, placed onto tissue, covered with parafilm, and hybridized at 62°C overnight in a humid chamber containing 50% 5X SSC/50% formamide. The slides were then rinsed in 4X SSC and digested with 20 μg/ml RNase (Sigma) for 30 min. at 37°C, followed by successive washes in 2X, 1X, and 0.1X SSC for 30 min. each at 65°C. Slides were then blocked for 15 min. (DIG wash and block buffer set, Boehringer Mannheim) and incubated with a sheep polyclonal anti-DIG antibody (Fab fragment, Boehringer Mannheim) conjugated to alkaline phosphatase used at 1:500 for 1 hr at r.t. After rinsing in Tris-buffered saline, treating with 20 mM levamisole (Vector Labs) for 30 min at r.t., and pre-equilibriating with detection buffer (Boehringer Mannheim), the slides were incubated in a substrate solution containing nitro-blue

tetrazolium-chloride (NBT 450 µg/ml, Boehringer Mannheim), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP 175 µg/ml, Boehringer Mannheim), and levamisole (2.5 mM) in detection buffer for 2-4 hrs, or until a purple reaction product was visible. For *in situ* hybridization combined with bromodeoxyuridine (BrdU) incorporation, mice (4 wpi and controls) were injected intraperitoneally (i.p.) with 200 mg/kg BrdU (Sigma) 4 and 2 hours prior to sacrifice. After the *in situ* hybridization detection was completed, slides were pretreated with 2N HCl for 30 min. at r.t., rinsed well in PBS, blocked as for immunocytochemistry (see above), and incubated overnight at 4°C in a humid chamber with a polyclonal anti-BrdU $F(ab')_2$ fragment antibody conjugated to horseradish peroxidase (diluted 1:15, Boehringer Mannheim). The slides were rinsed in PBS, and incubated with 3,3'-diaminobenzidine (DAB, Vector Labs) for 10 min. r.t., which forms a brown precipitate catalyzed by peroxidase. The slides were mounted with Glycergel (DAKO).

Data analysis

The hang time scores for each mouse were summed (seven time points over 4 wpi; 0, 7, 10, 14, 17, 22, 29 days post-injection) and compared to one another. For example, a mouse with no observed motor

deficit would have a summed score of 60 sec (the maximum limit) x 7 time points over 4 wpi = 420. Since clusters of mice with a mild deficit over 4 wpi, and with a severe deficit over 4 wpi existed, a median split was used to separate a mild and a severe group. Data from MHV-A59-injected, but behaviorally unaffected mice were not used for this analysis. To compare hang time scores or clinical scores from control, unaffected, mildly affected, and severely affected groups, a one way analysis of variance (ANOVA) with repeated measures was performed using Statview software. All graphs were made using Kaleidagraph software.

The weights of mice that survived the MHV-A59 injection were compared to the weights of mice that died on injection day with an unpaired Student's t-test (Statview software). The weights of control mice over time were compared to the weights of mildly impaired mice and severely impaired mice with a one way ANOVA with repeated measures (Statview software).

The number of cells expressing PDGF-A mRNA in remyelinating spinal cord (9 sections from 5 mice) *versus* control spinal cord (8 sections from 3 mice) were compared with an unpaired Student's t-test. The number of cells labeled with PDGFαR mRNA, nuclear BrdU incorporation, or both in remyelinating spinal cord (12 sections from 5

mice) *versus* control spinal cord (9 sections from 2 mice) were compared with an unpaired Student's t-test.

Chapter 3

Motor Behavior Associated with Demyelination and Subsequent Remyelination

A mouse model of experimentally induced acute demyelination followed by spontaneous remyelination was used to examine mechanisms involved in endogenous myelin repair. Mice given an intracranial (i.c.) injection of 1000 plaque forming units (PFU) of MHV-A59 coronavirus directly into the brain parenchyma first experience a viremic phase in which replicating virus can be isolated from brain and spinal cord, serum, and peripheral tissues (Lavi *et al.*, 1984). MHV-A59 lytically infects glial cells (Dubois-Dalq *et al.*, 1982) causing focal demyelinated lesions throughout the brain and spinal cord approximately 2 weeks post-injection (wpi) (Jordan *et al.*, 1989; Woyciechowska *et al.*, 1984). This pattern of focal lesions resembles the pattern of demyelination seen in multiple sclerosis. Viral transcripts in the spinal cord are found in focal areas of the white matter that is consistent with the pattern of demyelination observed by either Sudan black stain or by *in situ* hybridization for myelin-specific genes, such as myelin basic protein (Jordan *et al.* 1989a). Clinical symptoms of demyelination have been reported to occur from 1 to 4 wpi. The virus is then cleared from the CNS and periphery (Jordan *et*

al., 1989; Lavi *et al.*, 1984), and significant spontaneous remyelination occurs beginning approximately 3-4 wpi (Armstrong *et al.*, 1990; Jordan *et al.*, 1989; Jordan *et al.*, 1990; Kristensson *et al.*, 1986; Redwine and Armstrong, 1998). This mouse model is excellent for examining mechanisms that promote myelin repair that could ultimately be used as therapy to augment the limited endogenous remyelination that occurs in human diseases, such as multiple sclerosis (Prineas *et al.*, 1993; Raine and Wu, 1993).

Despite the advantages of this model, some reported inconsistencies need to be addressed. To improve and strengthen the value of the MHV-A59 model, data are presented in this chapter that address previously reported variability in mortality rates, and the frequency of mice reported to have neurologic symptoms. Variability in mortality rates is addressed by monitoring of mouse weights. Variability in reported percentages of mice with neurologic symptoms is addressed by developing objective criteria for neurologic symptoms with a motor test. This motor test was also used to estimate motor function improvement associated with histologic evidence of remyelination. The combined histologic and behavior data are then used to define a time point of early spontaneous myelin repair. Mechanisms involved in remyelination will be examined in

Chapters 4 and 5.

MHV-A59 titers

Plaque assays were used to verify virus stock concentration, and the concentration of diluted virus used for the i.c. injections. The stock virus provided by Kathryn Holmes (Dept. of Microbiology, University of Colorado Health Sciences Center) was harvested and frozen in 1993 and labeled as 2.1 x 10^8 PFU/ ml. According to a plaque assay performed in our laboratory on 17-Cl-1 mouse fibroblasts (kindly provided by Kathryn Holmes), the virus stock was 6.8 x 10^8 PFU/ ml, which is similar to the original concentration. Following serial dilutions from the stock vial to give 1000 PFU/ 10 µl injection volume, a "back-titration" plaque assay was performed to verify the accuracy of the dilutions. According to this plaque assay, the concentration of the dilution used for injections was 760 PFU/10 µl, which is very close to the desired 1000 PFU/ 10 µl concentration.

Mouse weight and mortality

Mortality following the MHV-A59 injection can be reduced by observing a minimum weight requirement.

There is variability in reported mortality rates among different

studies using the MHV model. Kristensson *et al.*, (1986) reported 70% mortality, while Armstrong *et al.* (1990) reported 11%. This variability may reflect other factors influencing survival outcome. To reduce mortality and to address the problem of mortality rate variability, a minimum weight requirement is specified, in addition to the previously established age criteria.

We documented the weight of mice on the day of the MHV-A59 injections. The weight of mice on the day of injection that eventually die following i.c. MHV-A59 injection was 12 ± 0.34 g (mean ± SE; n=25). The weight of mice on the day of injection that survive following i.c. MHV-A59 injections was 13.5 g ± 0.22 (mean ± SE; n=51). These two groups were statistically significantly different ($p < .0005$, unpaired student's t-test). Therefore, 28-day-old mice that weighed approximately 12 grams or less were more likely to die after infection than heavier 28-day-old mice given the same virus inoculation.

"Hang time" motor test

A motor test detects sub-groups of "mildly" and "severely" neurologically impaired mice following i.c. injection with MHV-A59.

Although this is an excellent model for examining mechanisms of myelin repair, different labs using this model have reported percentages of

mice with neurologic symptoms caused by demyelination that range from approximately 20% (Lavi *et al.*, 1984) to 75% (Jordan *et al.*, 1989). To address the problem of neurologic symptom variability, a motor test was developed to accommodate housing and testing restrictions relevant to this model. Available motor tests, such as the rotarod (Dunham and Miya, 1957; Kuhn *et al.*, 1995), have not been used in this model since the MHV-A59 injected mice require isolated housing. Contamination may also be a consideration for other models, such as CNS demyelination experimentally induced by Theiler's murine encephalitis virus (TMEV) (Miller *et al.*, 1995). The motor test developed here provides objective criteria for identifying neurologically impaired mice, and therefore may be used in other mouse models.

A "hang time" test was developed to monitor motor function. This motor test takes advantage of a natural behavior of mice, which is grasping the metal bars of a cage top when it is turned upside-down. Therefore, this test is easily administered, requires no apparatus, and does not require training of mice or learning of a new behavior.

Mice were suspended upside-down on cage tops held 18 - 24 inches from a table top (see methods). This suspension required the mice to hold on in order to prevent a fall. Control mice easily grip the cage top bars

with paws, often curl tails around the bars, and can move around and explore while upside-down (Figure 1a).

In contrast, mice with neurologic symptoms cannot grasp cage top bars with paws, rarely curl tails around bars, and fall off prior to the 60 second maximum time limit (Figure 1b and 1c). Inability to hold on is associated with demyelination as discussed in a later section, and as evident in previous reports of the histopathological damages in this model at 4-5 wpi (Armstrong *et al*, 1990; see Redwine and Armstrong, 1998). The time course of individual mouse motor performance over 4 wpi showed clustering of sub-groups, *i.e.*, a group of mice that had only mild motor dysfunction, and a group of mice that had severe motor dysfunction. This sub-group clustering may be another reason for previously reported variability with this model since mice from either group may have been combined in a given set of experiments. Operational definitions for mild and severe groups can be made two ways.

One method of distinguishing mild from severe groups used "summed hang times" over 4 wpi to derive a median split (Figure 2a). This method was used for statistical comparison of mild and severe groups. In addition to the median split criteria, other specifics of the hang time results criteria can be used to define mildly and severely affected mice. In

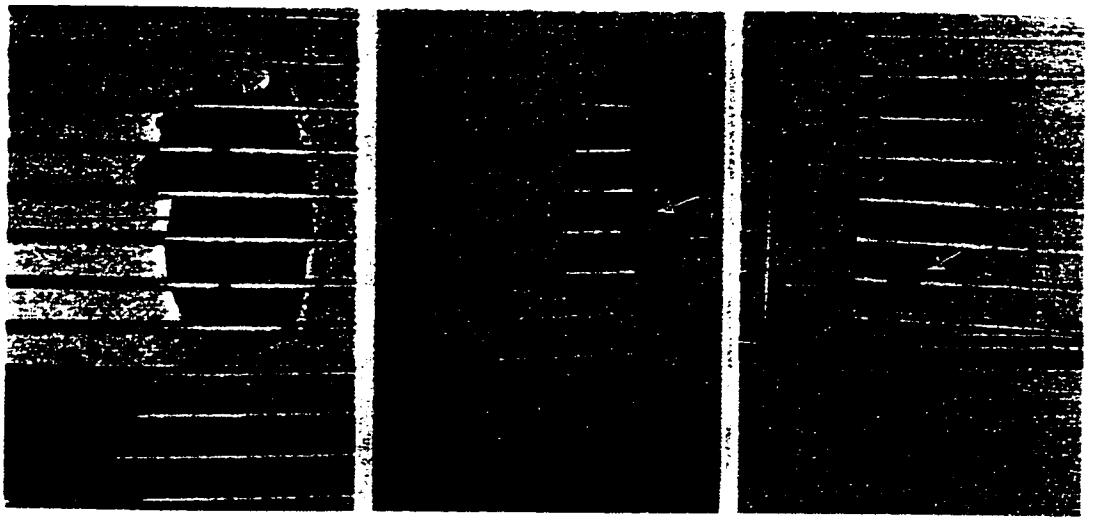


Figure 1. *Mice during the "hang time" motor performance test 3 wpi.* Control and impaired MHV-A59 injected mice are shown performing the hang time test at 3 wpi. PBS injected control mice support their weight while hanging upside-down on a cage top by grasping the cage top bars with toes (arrow in A), and often curl their tails around the bars while moving around (arrowhead in A). Severely impaired mice injected with MHV-A59 are often unable to grasp the cage top bars with their toes (arrow in B and C), and typically do not curl their tails around the bars (arrowheads in B and C) during early time points following the MHV-A59 injection (1-3 wpi). The ability to grasp the cage top bars returns at later time points (not shown).

practice, we found that mildly affected mice either a) completely recover to baseline by 14 dpi, or b) have motor deficits never worse that 50% of the maximum value for the test (*i.e.*, always above 30 sec). Severely affected mice have motor deficits persisting beyond 14 dpi, and perform worse than 50% of the maximum value at any one time point (*i.e.*, below 30 sec; Figure 2b). All mice (n=25), except for one, could be consistently categorized as mild or severe with either median split analysis or with the above behavioral criteria. However, whereas the median split analysis requires summed values over time, the behavioral criteria are useful for identifying mild or severe mice that need to be selected for analysis and sacrificed at early time points in the disease progression.

When groups are separated using a median split, there is a clear statistical difference between the mild and severe groups. Figure 3a shows the average hang times over 4 wpi of mildly affected *versus* severely affected mice. The mild group means (mice above the median split) demonstrate significantly less impairment over time compared to severe group means (mice below the median split), determined with a one way analysis of variance (ANOVA) with repeated measures ($p< .0001$).

The hang time test also detects functional recovery following i.c. MHV-A59 injection.

An improvement in neurologic function or recovery of function during the remyelination phase has never been quantified in the MHV-A59 model. This recovery is important to demonstrate in order to show the clinical relevance of the remyelination. The hang time test used to objectively identify neurologic impairment enables quantification of motor function over time, and can therefore be used to quantify motor recovery. Figure 3a shows that the motor performance of severely affected mice significantly improves by 4 wpi compared to earlier time points, such as 1.5 wpi. Therefore, the hang time test is sensitive enough to detect individual differences over time that previously have not been clearly reported or quantified.

A clinical score scale is not as sensitive as the hang time test for detecting neurologic impairment or recovery.

The results of the hang time test were compared to the commonly used clinical score scale (modified from Cross *et al.*, 1994; Lannes-Vieira *et al.*, 1994; Lavi *et al.*, 1984; Miller *et al.*, 1992) for the same groups of mice. The clinical score scale is a measure of impairment based upon visible limb dysfunction (see methods). The clinical scores of mildly affected mice over time are significantly less than the clinical scores of

severely affected mice (Figure 3b). The clinical score scale is used in other models, and supplements the hang time motor test. However, it does not detect functional improvement as can be ascertained from the hang time test (compare Figure 3a with 3b). In addition, the limited unit range of the clinical score scale does not detect individual variation as well as the hang time motor test. When the hang times and clinical scores of individual mice are compared, the sensitivity of the hang time test is evident (compare Figure 3c with 3d).

Complete functional recovery occurs after MHV-A59 injection.

Although most mice were sacrificed at 4 wpi, 100% of mice monitored beyond 4 wpi fully recovered functionally (*i.e.*, maintained 60 second hang times) by 6.5 wpi (n=5) (Figure 4).

Mouse weight following MHV-A59 injection

The weights of severely impaired mice are significantly lower than the weights of mildly impaired mice, unaffected mice, or control mice over 4 wpi when compared with an ANOVA with repeated measures (Figure 5). The weights of mildly impaired mice are significantly less than the weights of unaffected mice or control mice over 4 wpi when compared with an ANOVA with repeated measures (Figure 5).

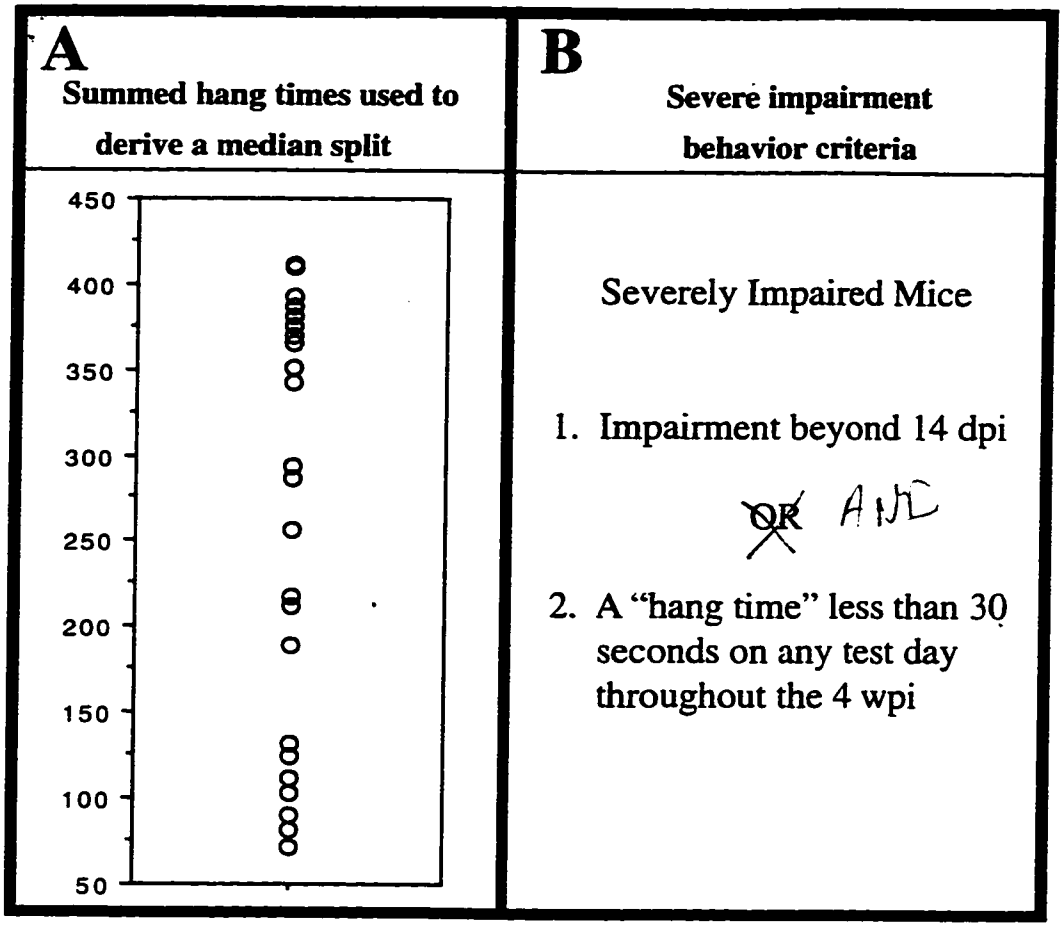
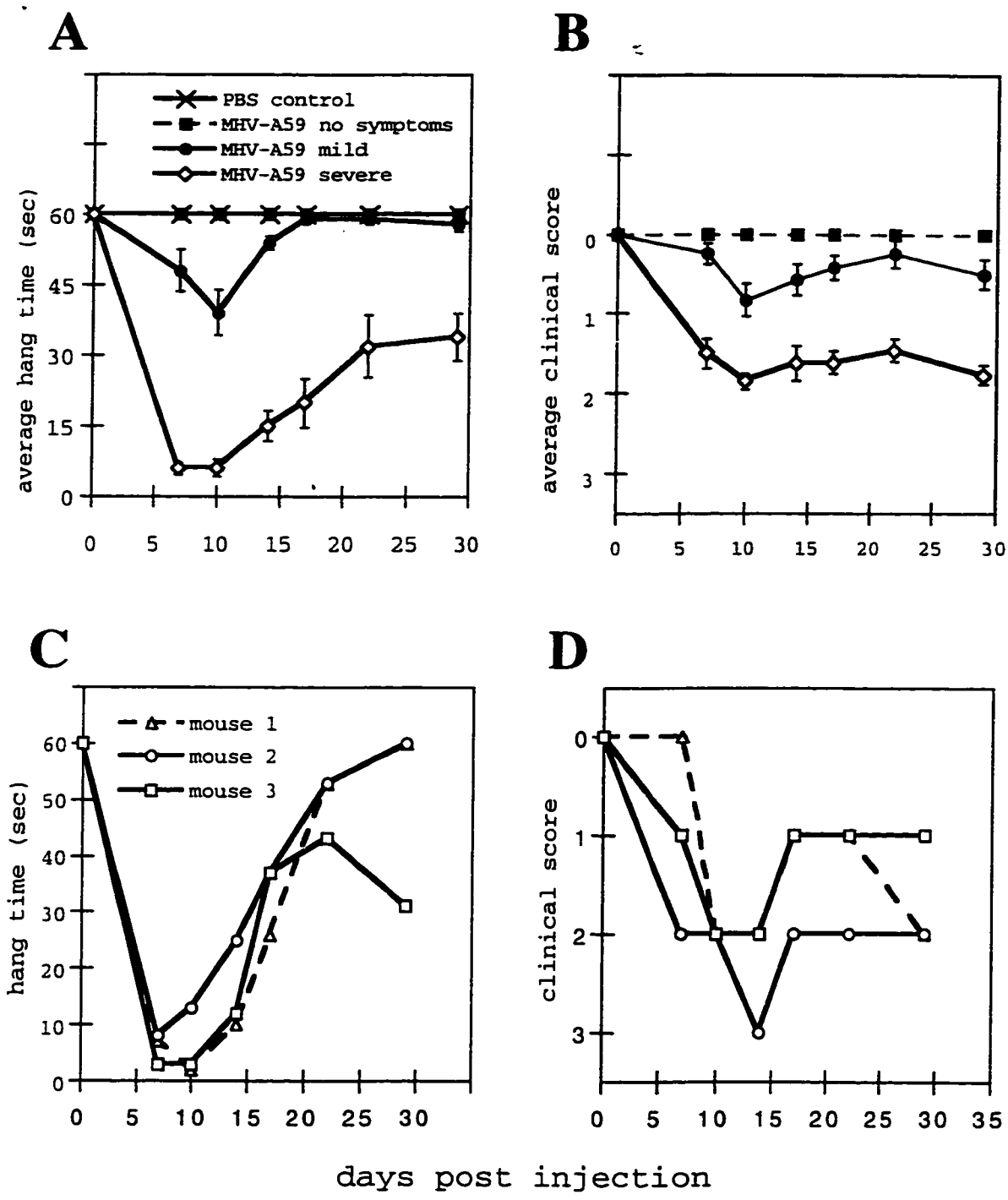


Figure 2. *Criteria for mildly and severely affected mice.*

The hang times for each mouse with motor impairment (seven time points over 4 wpi; 0, 7, 10, 14, 17, 22, 29 dpi) were summed and are compared to one another in panel (A) (n = 25). Clusterings of subgroups that have either low summed hang times or high summed hang times are evident. A median split was used to separate severely impaired mice (low summed hang times) and mildly impaired mice (high summed hang times) (median split = 320). Hang times are shown over time in Figure 3. Panel (B) states behavioral criteria also used to identify mild versus severe motor impairment.

Figure 3. *Hang times and clinical scores of control and MHV-A59 injected mice throughout the first 4 wpi.*

The hang times (A) and clinical scores (B) of severely impaired mice (n = 13), mildly impaired mice (n = 12), unaffected mice (n = 7), and controls (n = 10) are shown over 4 wpi. ANOVA with repeated measures verifies that severely impaired mice have significantly lower hang times ($p <$ .0001) and clinical scores ($p <$ .0001) than the mildly impaired, unaffected or control mice. To show the increased sensitivity of the hang time test compared to the clinical score scale, the hang times and clinical scores of three individual mice are shown (C,D). The time course of motor impairment followed by motor recovery is detectable in individual mice when using the hang time test (C), but this progression is not as evident in individual mice when using the clinical score scale (D).


A
PBS control
MHV-A59 no symptoms
MHV-A59 mild
MHV-A59 severe
average hang time (sec)
0
15
30
45
60
0 5 10 15 20 25 30
B
average clinical score
0
1
2
3
0 5 10 15 20 25 30
C
mouse 1
mouse 2
mouse 3
hang time (sec)
0
10
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50
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clinical score
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days post injection

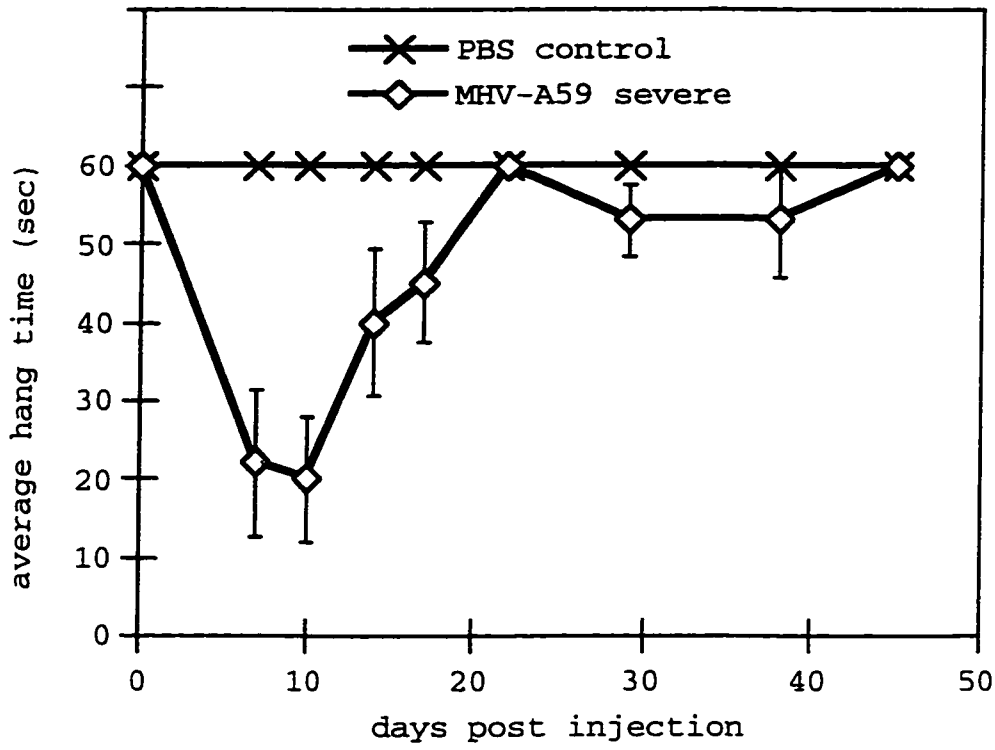


Figure 4. *Motor recovery following the MHV-A59 injection.*

Hang times of severely impaired mice (n = 5) are shown throughout the first 6.5 weeks following the MHV-A59 injection. All mice tested recovered completely by 45 dpi.

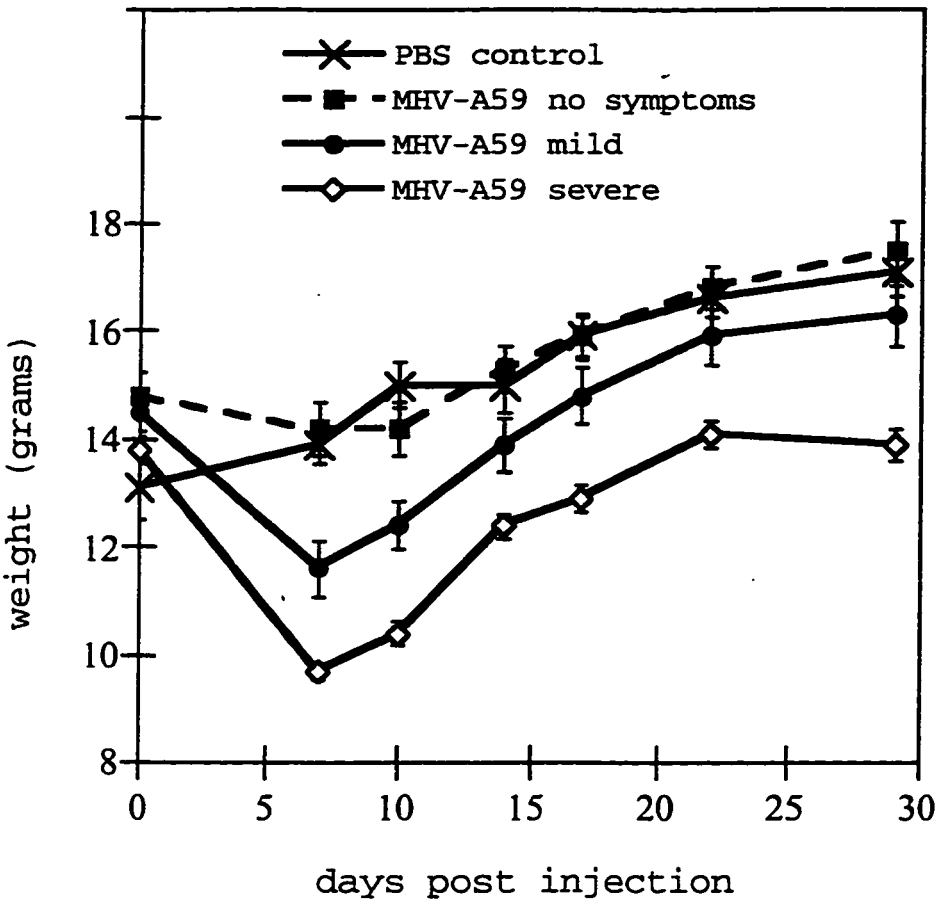


Figure 5. *Mouse weights following the MHV-A59 injection.*

Mouse weights were monitored throughout the first 4 wpi following the MHV-A59 injection. The average weights of severely impaired mice (n = 13), mildly impaired mice (n = 12), unaffected mice (n = 7), and PBS injected control mice (n = 10) are shown. ANOVA with repeated measures verifies that severely impaired mice weigh significantly less over time than mildly impaired or control mice ($p < .0001$).

Spinal cord myelin following MHV-A59 injection

Reduced immunoreactivity for myelin protein in MHV injected mice at 4 wpi.

Figure 6a shows reduced immunoreactivity for two isoforms of proteolipid protein (PLP) within a cervical dorsal column lesion compared to surrounding non-lesioned white matter. Other studies have reported reduced immunoreactivity for other myelin proteins at similar time points (Jordan *et al.*, 1990).

Histologic evidence of remyelination at a time point that corresponds to motor function improvement.

At 4 wpi, clear demyelination with evidence of remyelination, such as thin myelin sheaths, was observed by light microscopy (Figure 7b,d). Remyelination progresses over the following weeks so that areas of demyelination are efficiently repaired (Jordan *et al.*, 1989; Godfraind *et al.*, 1989; Armstrong *et al.*, 1990b), and stable myelin is maintained in remyelinated areas six months after MHV-A59 injection (Figure 7c).

Figure 6. *Myelin-specific protein immunoreactivity is reduced in lesions.*

A mouse spinal cord (4 wpi) was immunolabeled with a rat monoclonal antibody that recognizes developmental (DM20) and mature isoforms of proteolipid protein (PLP), a myelin-specific protein. Figure 6 shows varying degrees of myelin protein loss. Significantly reduced PLP/DM20 immunoreactivity is observed within a dorsal column lesion above the white line to the right. White matter with less myelin protein loss but with vacuolization, increased cellular infiltration, and tissue disruption is observed above the white line to the left, and below the white line to the left of the white matter area. These areas represent varying degrees of demyelination compared to surrounding corticospinal tract white matter (below the white line to the right of the white matter area) (A). A phase contrast image shows vacuolated, reactive tissue within the lesion compared to non-lesioned tissue (B). WM = white matter; GM = gray matter.

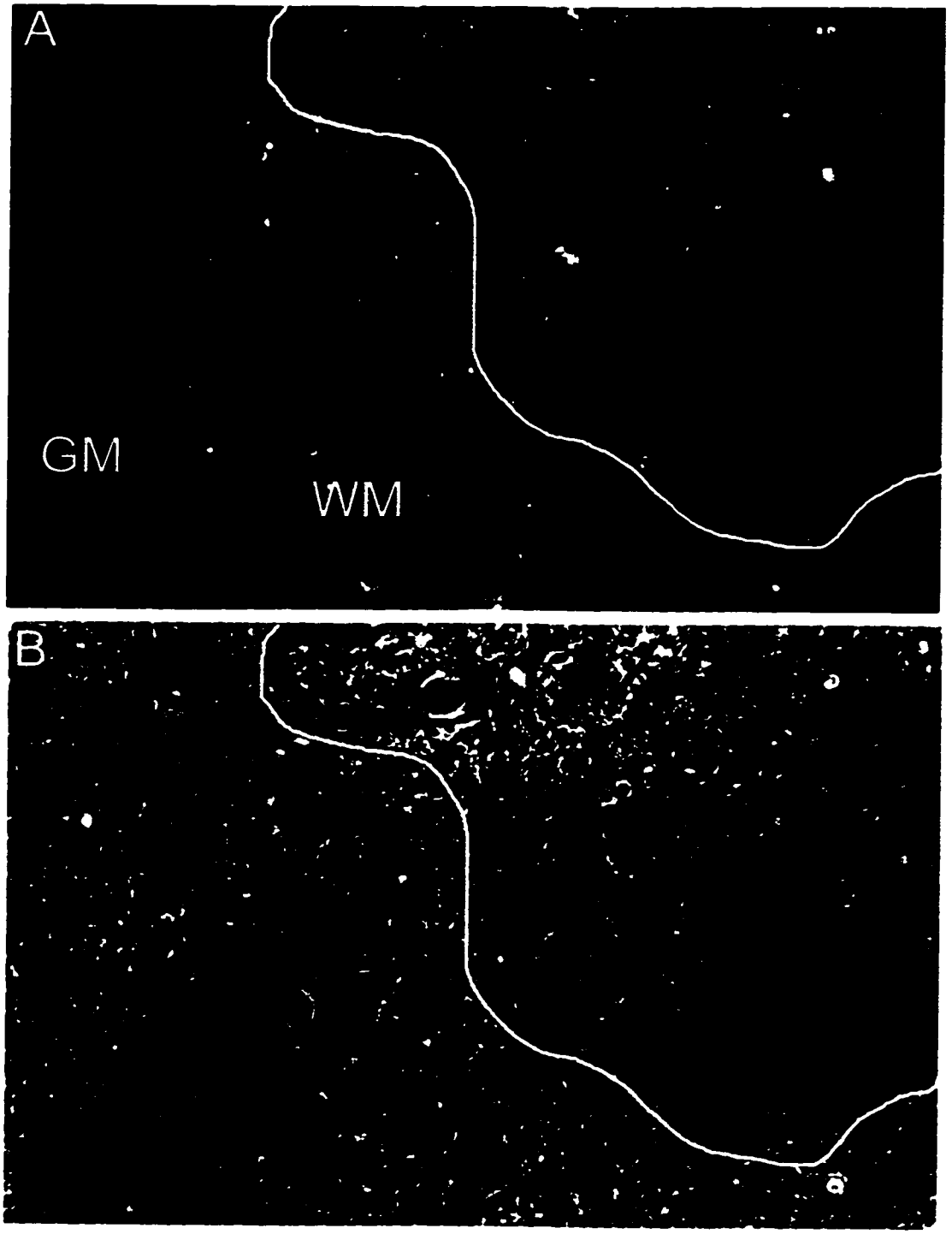
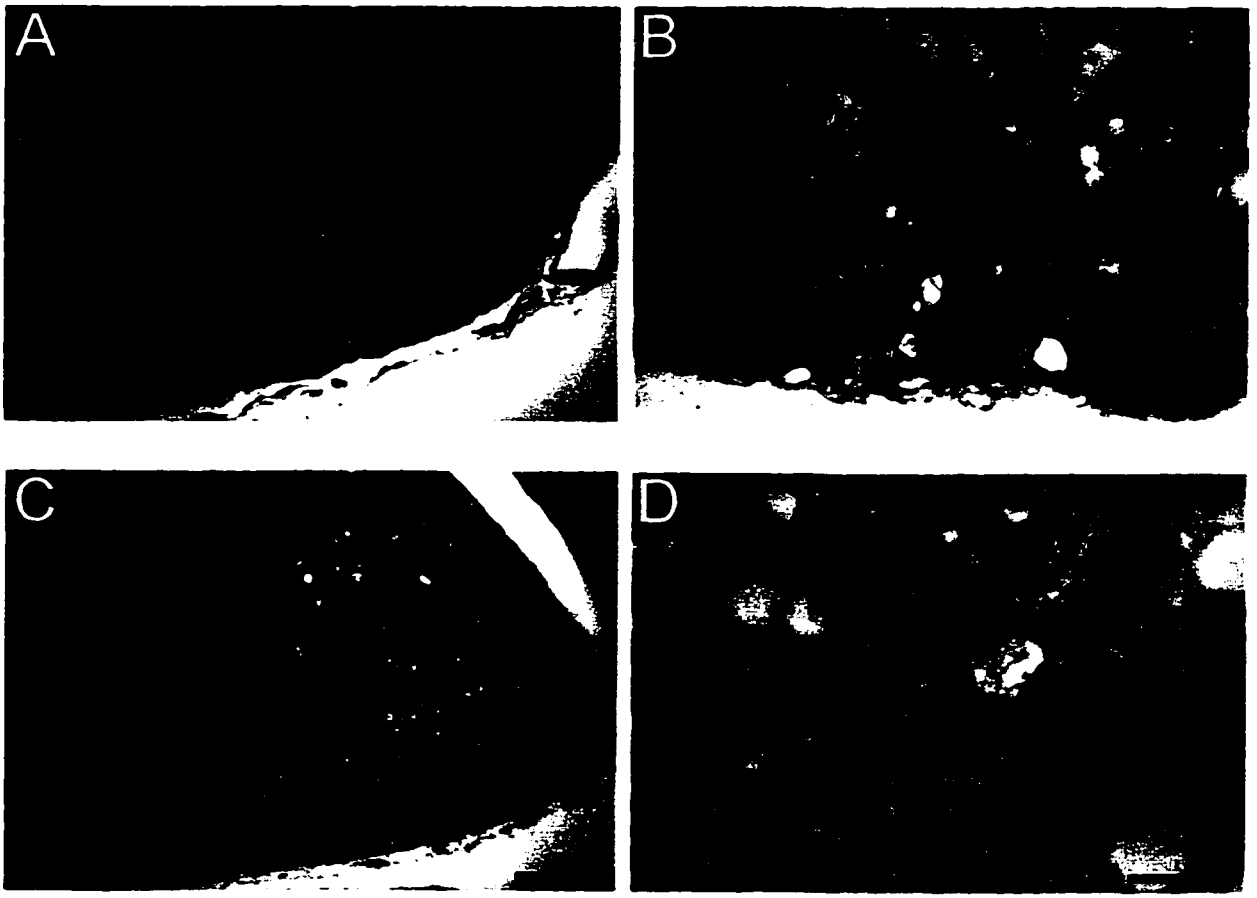

A
GM
WM
B

Figure 7. *Histologic evidence of remyelination.*

White matter from the ventrolateral quadrant in transverse sections of cervical spinal cord segments from MHV-A59 injected mice. Non-lesioned white matter is shown in (A), compared to lesioned white matter at 4 wpi (B and D) and remyelinated white matter at 6 months post injection (C). At 4 wpi (B), the lesion has areas of increased cell density as well as vacuolization. Higher power of the central area of the 4 wpi lesion (D) shows very thin myelin sheaths, consistent with early remyelination. At 6 months post injection (C), an area of remyelinated ventral white matter lesion is apparent by comparing the thin myelin sheaths toward the right of panel (C) to the myelin sheaths with normal thickness to the left of panel (C) and to the non-lesioned ventral white matter in panel (A). A-C scale bar = 200 μm, D scale bar = 50 μm.


A
B
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D

Discussion

A behavior test was developed to monitor motor deficits associated with previously reported demyelination (Jordan *et al.*, 1989; Woyciechowska *et al.*, 1984), and to monitor subsequent functional improvement and/or recovery associated with previously reported remyelination (Armstrong *et al.*, 1990; Jordan *et al.*, 1989; Kristensson *et al.*, 1986; Redwine and Armstrong, 1998). A similar test, independently developed by Sango *et al.* (1996), monitored mouse behavior of grasping the wires of a cage top and detected motor deficits in mice lacking α and β subunits of the lysosomal enzyme β-hexosaminidase. The advantages of the "hang time" motor test are 1) it takes advantage of a natural motor behavior, so training and learning are not involved, 2) this test does not require equipment or highly skilled personnel, 3) this test provides objective reproducible criteria for identifying mice with motor impairment, and 4) this test provides an objective and sensitive method of motor performance of individual mice throughout disease progression. This assay is very valuable for models such as this one, since time points corresponding to severe dysfunction, the beginning of functional improvement and complete functional recovery can be detected for individual mice.

By using the hang time motor performance test we identify time points of severe motor dysfunction in mice injected with MHV-A59, and more importantly for this model, time points of motor function improvement and recovery following MHV-A59 injection. To date, motor function improvement and/or recovery has not been assessed quantitatively in this model. In addition, we showed that the sensitivity of the hang time measure enabled identification of two subgroups of neurologically affected mice: a mildly impaired group and a severely impaired group.

The common method of scoring the number of affected appendages, severely impaired mice were assigned significantly higher clinical scores (meaning worse impairment) than mildly impaired mice. However, the clinical score scale, when used alone, is not easily reproducible, since the identification of an affected limb depends upon the experience and judgement of individual investigators. Also, it is not as efficient as the hang time test at detecting individual differences or improvement in motor function over time, due to the limited score increments.

Identification of mildly and severely affected mice following the i.c. MHV-A59 injection is useful since 1) subpopulations of mildly and severely affected mice can be further analyzed as more homogeneous individual groups, and 2) identification of two groups may reduce variability among laboratories. Woyciechowska *et al.* (1984) noted several mice recovered

early after MHV-A59 infection, but no behavioral criteria for identifying early recoverers were given. Woyceichowska *et al.* (1984) documented certain neurologic symptoms that can be observed behaviorally, however other studies using this model have not clearly documented criteria for identifying the extent of motor impairment. Lavi *et al.* (1984) reported mice were "mildly", "moderately", or "severely" sick following i.c. MHV-A59 injection without employing quantitative or statistical measures. In contrast, Jordan *et al.* (1989) reported 75% of similarly injected mice had neurologic symptoms, often including hindlimb weakness or paresis. The reasons for previously reported variability may be the lack of consistent and objective monitoring of neurologic symptoms, and the existence of two significantly different subgroups of impaired mice that have not been previously defined in this model.

The weights of severely impaired, mildly impaired, and unaffected mice are significantly different from one another over the first 4 wpi. This data may indicate indirectly that the severity of motor impairment, and therefore demyelination, is dependent on the severity or extent of the initial lytic viral infection in each mouse.

A prerequisite for injecting mice in this model was an age requirement of 28 days. Intracranial MHV-A59 injections at this age cause

demyelination in a significant percent of animals injected and lead to a reduced percent of mortality compared to viral injections in younger mice (Kathryn Holmes, personal communication). In addition, the heaviest 28-day-old mice were found to survive better in some previous experiments (Kathryn Holmes, personal communication). However, previous studies have not reported weight criteria at the time of injection, and reported mortality rates have ranged from 11% (Armstrong *et al.*, 1990) to 70% (Kristensson *et al.*, 1986). If 28-day-old mice weighing 13 grams or more at injection time are used exclusively (instead of 28-day-old mice that weigh 9-12 grams), high mortality rates such as reported in Kristensson *et al.* (1986) may have been avoided. Therefore, in addition to the 28-day-old age requirement previously used, a minimum weight requirement of 13 grams should also be used to reduce mortality rates in this model.

Using a minimum weight criterion to minimize mortality as well as having the hang time test as a simple, reproducible means of quantifying disease progression will make MHV-A59 infection a more useful model of experimental demyelination and remyelination. The capability of monitoring motor impairment over time should be beneficial to other mouse models, such as models of EAE that involve episodes of demyelination (Dal Canto *et al.*, 1995; Miller *et al.*, 1995, Raine *et al.*,

1988), mouse myelin mutants (Kuhn *et al*, 1995), or mouse models of neuronal degeneration (Collard and Julian, 1995).

Chapter 4

Oligodendrocyte Progenitors in Normal and Remyelinating Mouse Spinal Cord

The motor performance and histologic data presented in Chapter 3 are used to identify a time point corresponding to early remyelination following the i.c. MHV-A59 injection. At 4 wpi, mice display an improved motor performance compared to earlier time points, and showed histological evidence of remyelination. Therefore, the 4 wpi timepoint was selected as an early stage of remyelination to examine OLC responses associated with lesion repopulation.

Adult oligodendrocyte progenitors in normal mouse spinal cord

In normal tissue, adult oligodendrocyte progenitors have been identified with either an NG2 antibody (Levine and Stallcup, 1987; Levine *et al.*, 1993) or an antibody against PDGFαR (as discussed in Chapter 1), and have been found throughout the white matter and gray matter of the mouse spinal cord. The progenitor phenotype of NG2 positive (NG2+) cells was confirmed by double or triple immunolabeling to demonstrate the absence of either GFAP, an astrocyte marker (Figure 8), or Mac-1, a microglial cell marker (Figures 13 and 14; see Nishiyama *et al.*, 1996,

1997; Reynolds and Hardy, 1997). NG2 and PDGFαR have previously been shown to be expressed simultaneously on oligodendrocyte progenitors in adult rat CNS (Nishiyama *et al.*, 1996). In the adult mouse, the NG2+ or PDGFαR+ cells appeared to have the same morphology and distribution, but double immunolabeling was not tested because of the technical difficulty that both NG2 and anti-PDGFαR are polyclonal rabbit IgG antibodies and cannot be used simultaneously.

Adult oligodendrocyte progenitors express growth factor receptors PDGFαR and FGFRs in normal tissue.

To detect bFGF receptors, monoclonal antibody VBS1 was used, that recognizes mainly FGFR1 with some reactivity with FGFR2 (Venkateswaran *et al.*, 1992). This specificity appears to be consistent with preliminary analysis of mouse tissue using rabbit polyclonal antibodies that distinguish FGFR1, FGFR2, and FGFR3 (Regina Armstrong, unpublished observation). However, in mouse spinal cord, the only effective immunolabels for adult oligodendrocyte progenitors are the rabbit polyclonal antibodies against NG2 and PDGFαR which cannot be used without nonspecific cross-reactivity occurring with the rabbit polyclonal antibodies for each of the FGFRs.

In normal mouse spinal cord, oligodendrocyte progenitors

recognized by NG2 or PDGFαR exhibit variable levels of intensity of immunoreactivity for FGFRs. For example, adult oligodendrocyte progenitors often express FGFRs (Figure 8), but not always, as shown in Figure 9. Typically, the FGFR immunoreactivity was most intense in the cell body and was only found to a small extent on cell processes, in contrast to NG2 and PDGFαR immunoreactivity that was present along the cell body, but also extended out into the fine processes which are characteristic of the oligodendrocyte progenitor cells. The overall intensity of FGFR immunoreactivity in a given progenitor cell varied markedly between cells, even within the same area of tissue, indicating the possibility of *in situ* modulation of FGFR expression level among oligodendrocyte progenitors (Figures 8,9). This variability of FGFR expression did not correlate with localization within the spinal cord, so that the range of immunolabeling intensity was similar in gray matter and white matter.

Cells in the subventricular zone (SVZ), which are thought to be neuronal and glial progenitor cells that persist into adult animals also express PDGFαR and FGFRs (Figure 10).

Other cell types express PDGFαR and FGFR in normal tissue.

In normal mouse spinal cord adult oligodendrocyte progenitors

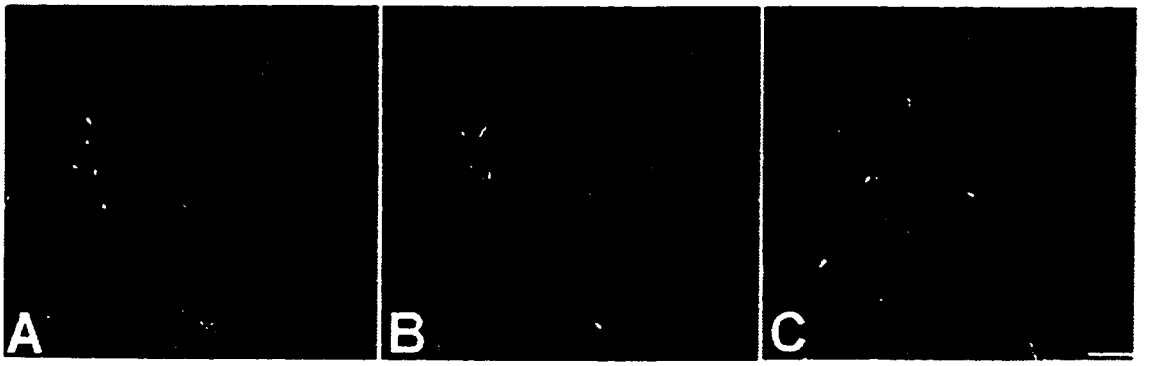
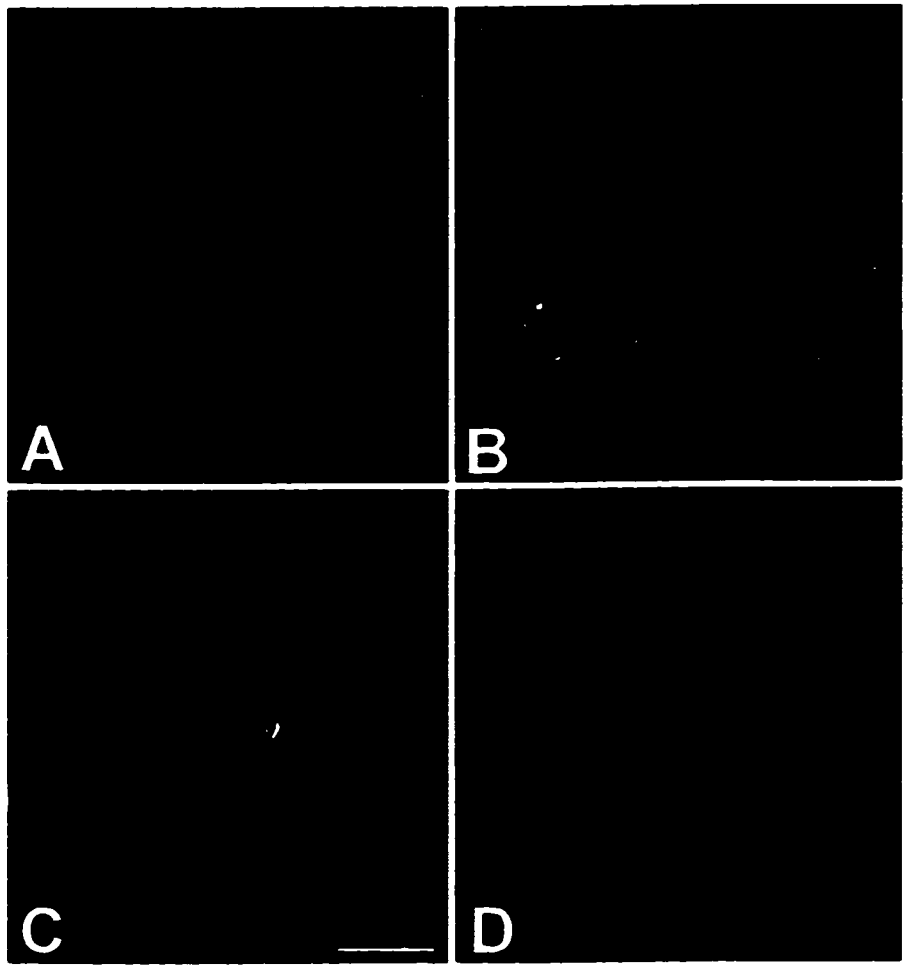


Figure 8. *FGFR immunoreactivity in oligodendrocyte progenitors.*

A-C: White matter in a transverse section of normal spinal cord from an 8 week-old mouse (age matched for 4 wpi remyelinating tissue) is shown after triple immunolabeling with anti-NG2 (A), anti-FGFR (B), and anti GFAP (C). The NG2+/GFAP- oligodendrocyte precursor cells have fine processes which extend in three dimensions (see Figure 14A) so that only a small extent of processes is shown in this single 0.25 μm optical section through the cell bodies. Anti-FGFR immunoreactivity (B) predominantly localizes to the cell bodies of the NG2+ oligodendrocyte progenitor cells (A). Immunohistochemistry shown in this figure was performed by Karen Blinder, and the CELLscan image was acquired by Regina Armstrong. A, C: CELLscan images acquired with a 63X objective and deconvolved; scale bar shown in C = 10 μm.

Figure 9. *Biological variation of FGFR immunoreactivity intensity* . A-D: Gray matter in a transverse section of spinal cord from an 8-week-old mouse is shown after triple immunolabeling with anti-PDGFαR (A), anti-FGFR (B), and anti-GFAP (C). A-D are three-dimensional reconstructions of each stack of 33 optical sections collected at 0.25 μm apart. D: The merged three-dimensional reconstruction color image shows the lack of marked colocalization of PDGFαR (A converted to red), FGFR (B converted to green), and GFAP (C converted to blue) immunoreactivity. Neurons typically exhibit intense cytoplasmic FGFR immunoreactivity (B,D). The PDGFαR+ oligodendrocyte progenitor is GFAP- and exhibits only minimal detectable FGFR immunoreactivity in this example (A,B,D), although other oligodendrocyte progenitors generally exhibited much more intense FGFR immunoreactivity (see Figure 8). Astrocytes exhibited a similar variation in intensity of FGFR immunoreactivity, with intense signal most evident in the radial astrocytes (see Figure 11), while signal was not detectable in occasional stellate astrocytes (B,C,D). A-D: CELLscan images acquired with a 63X objective and deconvolved; image stacks merged as color mixes of red, green, and blue intensities using Metamorph (image acquisition done by Regina Armstrong); scale bar shown in C = 10 μm.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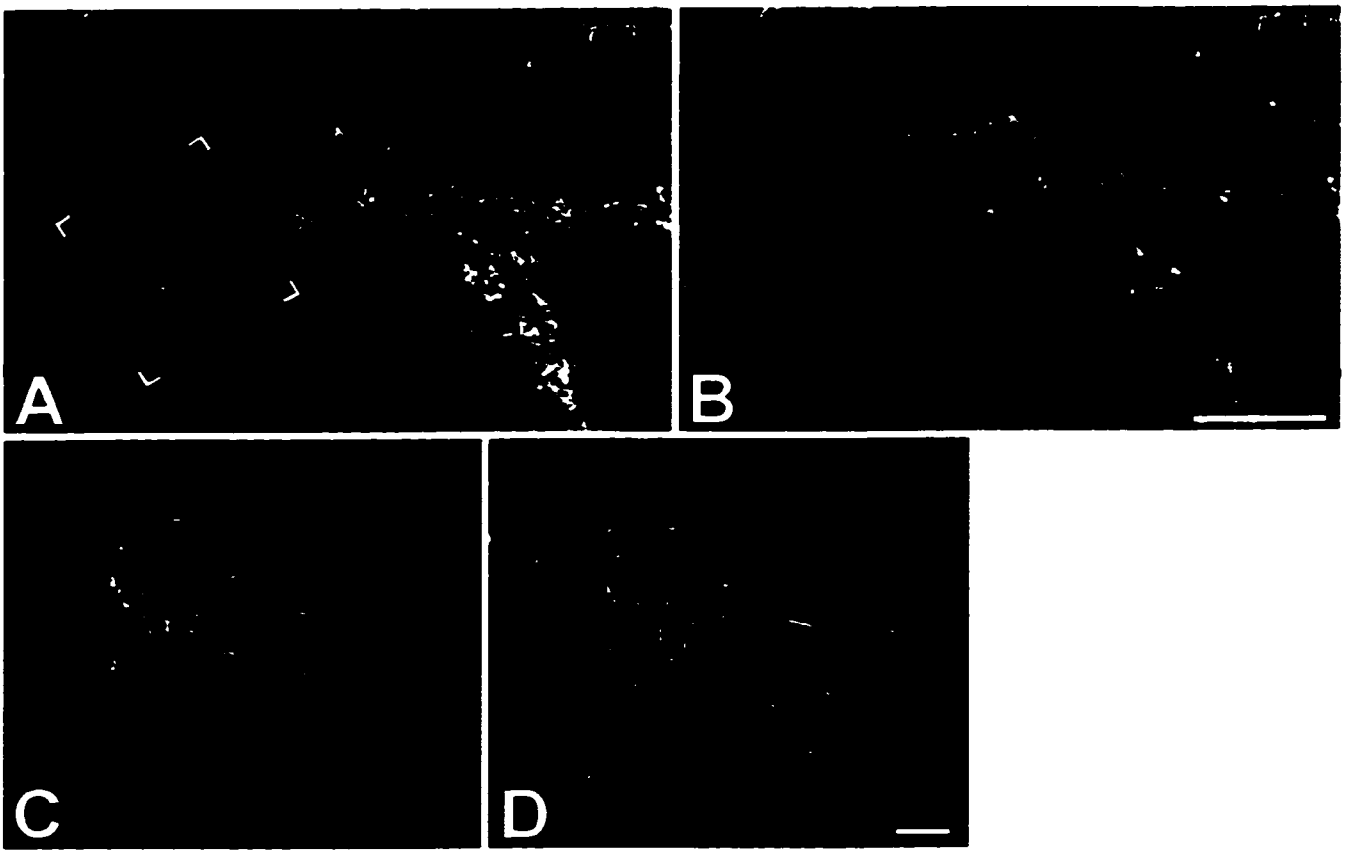
D

Figure 10. *FGFR and PDGFαR colocalization in subventricular zone cells.* A,B: A coronal section of forebrain from an 8-week-old mouse is shown after double immunolabeling for PDGFαR (A) and FGFR (B). The small, densely packed immunolabeled cells within and extending laterally from the subventricular zone (edges denoted by double-headed arrow in A) have the characteristic distribution and morphology of neural precursor cells. The area bracketed in (A) is reoriented and shown as single high resolution 0.25 μm optical sections for PDGFαR (C) and FGFR (D). A,B: Images acquired with a 25X objective, no deconvolution; scale bar shown in (B) = 50 μm. C,D; CELLscan images acquired with a 63X objective and deconvolved, scale bar shown in (D) = 10 μm.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express PDGFαR and FGFRs. Other cell types also express FGFRs and PDGFαR.

Some large ventral spinal cord motor neurons were immunoreactive for PDGFαR, although the fluorescent intensity was much lower in neurons than in adult oligodendrocyte progenitors (not shown since low level of immunoreactivity made photography difficult; see also Oumesmar *et al.*, 1997). Not all neurons were PDGFαR immunoreactive (Figure 9).

Neurons (Figure 9) and GFAP+ astrocytes (Figure 11) in normal adult mouse CNS, including the spinal cord, are immunolabeled by the VBS1 antibody against FGFRs.

Adult oligodendrocyte progenitors during remyelination

Increased immunoreactivity for oligodendrocyte progenitor markers NG2 and PDGFαR within and near lesions.

At 4 wpi with MHV-A59, increased immunoreactivity for NG2 and PDGFαR is present in and near areas of demyelination relative to adjacent normal white matter (Figure 12). The NG2+ or PDGFαR+ cells have small, elongated cell bodies and extend thin branched processes that become less extensive on the reactive cells within and near lesions (Figures 13,14,15).

NG2 retains oligodendrocyte progenitor specificity in remyelinating, reactive tissue.

Multilabeling shows that reactive NG2+ cells are oligodendrocyte progenitors (Levine *et al.*, 1994; Nishiyama *et al.*, 1997; Keirstead *et al.*, 1998), which are not immunolabeled with Mac-1, a marker for microglia, or for GFAP, a marker for astrocytes (Figures 13,14).

Adult oligodendrocyte progenitors express PDGFαR and FGFRs during remyelination.

In remyelinating mouse spinal cord (4 wpi), reactive oligodendrocyte progenitors recognized by NG2 or PDGFαR almost always have immunoreactivity for FGFRs within and near demyelinated lesions (Figure 15).

Complexity of cellular response during remyelination

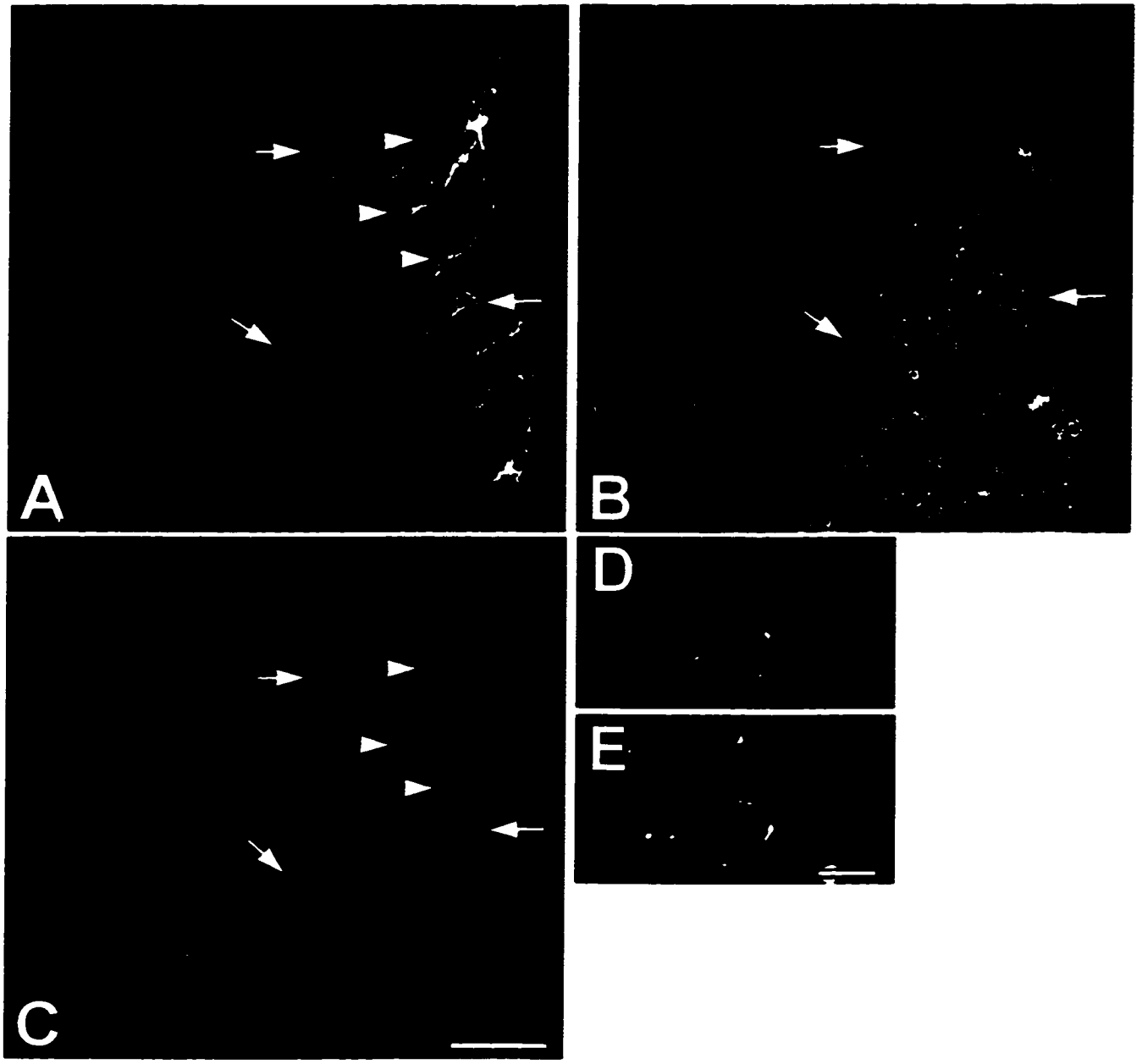
In addition to the increased immunoreactivity for oligodendrocyte progenitor markers within and near lesions, there is an increase in immunoreactivity for other cell phenotype markers within and near lesions. Microglia-specific immunoreactivity (Mac-1; Figure 13) and astrocyte-specific immunoreactivity (GFAP; Figure 14) are increased

specifically within and near remyelinating lesions at 4 wpi.

In contrast to normal mouse spinal cord, where only adult oligodendrocyte progenitors express PDGFαR, large reactive astrocytes in demyelinated lesions gained immunoreactivity for PDGFαR (Figure 15). Therefore, triple immunolabeling was used to clearly demonstrate that in normal and in lesioned white matter PDGFαR+ cells, with small elongated cell bodies similar to NG2+ cells, were also immunolabeled for FGFRs, but were not immunolabeled for the astrocyte marker GFAP (Figure 15).

In addition to the local glial response during remyelination, there is an infiltration of round immune cells within and near lesions. They were discovered when remyelinating tissue was incubated with fluorochrome-conjugated $F(ab')_2$ fragment antibodies against mouse IgM or IgG. These antibodies bound to endogenous mouse immunoglobulin. The presence of mouse immunoglobulin on the surface of round cells suggests that these cells were immunoglobulin-producing B lymphocytes (not shown). An antibody against a pan-B cell marker, CD45 receptor, labeled round cells within and near lesions, confirming the presence of cells with a B lymphocyte phenotype (data not shown). A more complete characterization of immune cells within mouse spinal cord during demyelination or remyelination in this model is warranted.

Figure 11. *FGFR immunoreactivity in oligodendrocytes and astrocytes.* A-C: White matter in a transverse section of spinal cord from an 8-week-old mouse is shown after triple immunolabeling with anti-FGFR (A), anti-CNP (B), and anti-GFAP antibody (C). As noted by arrows, several CNP+ oligodendrocytes (B) exhibit FGFR immunoreactivity (A). As noted by arrowheads, several GFAP+ astrocytes (C) exhibit FGFR immunoreactivity (A). D,E: In a coronal section of 8-week-old mouse brain, a CNP+ oligodendrocyte (D) in the striatum is shown to have FGFR immunoreactivity (E) which colocalizes mainly to the oligodendrocyte cell body in each single 0.25 μm optical section. A-C: Images acquired with a 25X objective, no deconvolution; scale bar shown in C = 50 μm. D,E: CELLscan images acquired with a 63X objective and deconvolved; scale bar shown in E = 10 μm.


A
B
C
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E

Figure 12. *NG2 and PDGFaR immunoreactivity within and near remyelinating lesions 4 wpi with MHV-A59.*

Increased NG2 immunoreactivity (A) is evident in an area of demyelination (B), which lacks myelin birefringence by darkfield imaging (normal myelin appears white in B,D). Increased PDGFαR immunoreactivity (C) is evident in an area of demyelinated white matter (D). Scale bar = 200 μm.

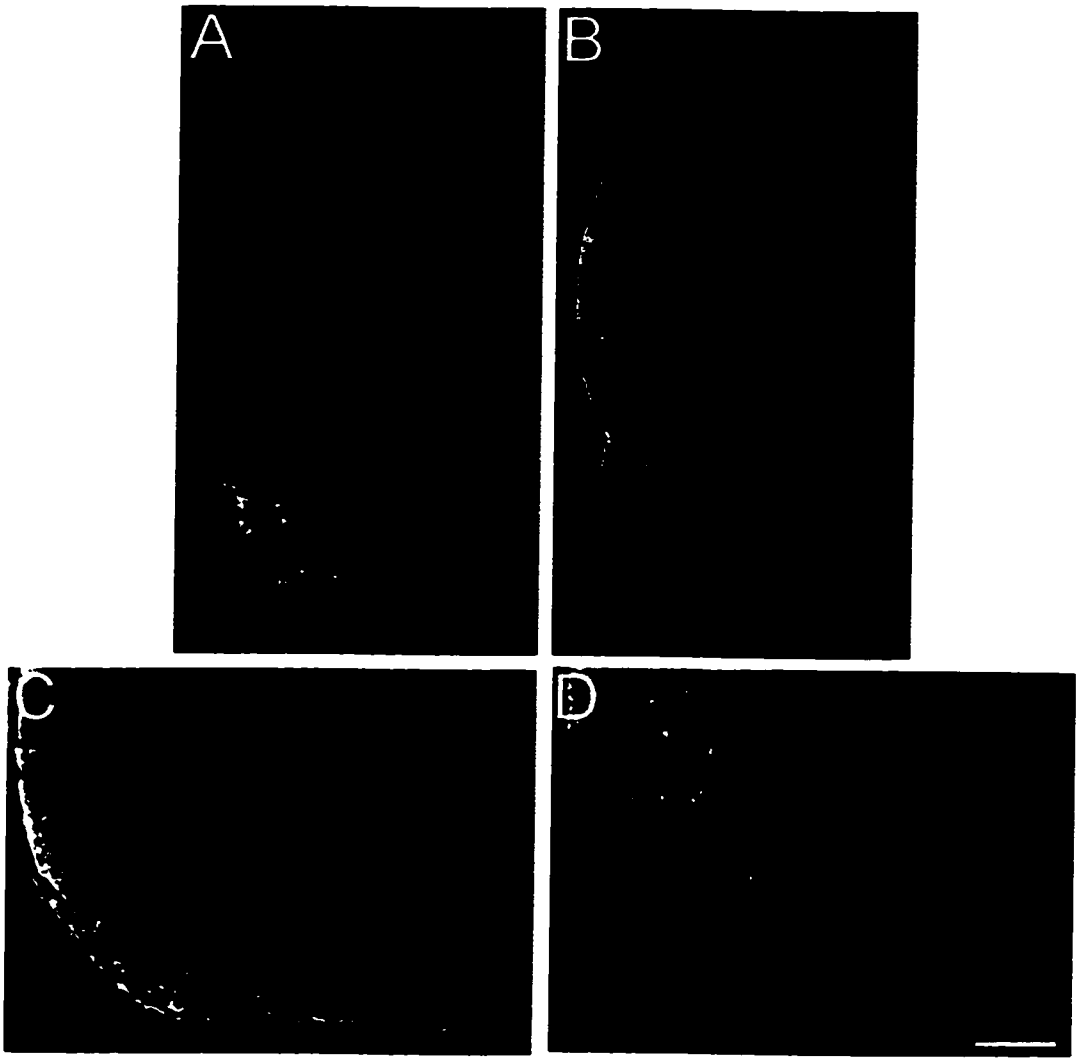
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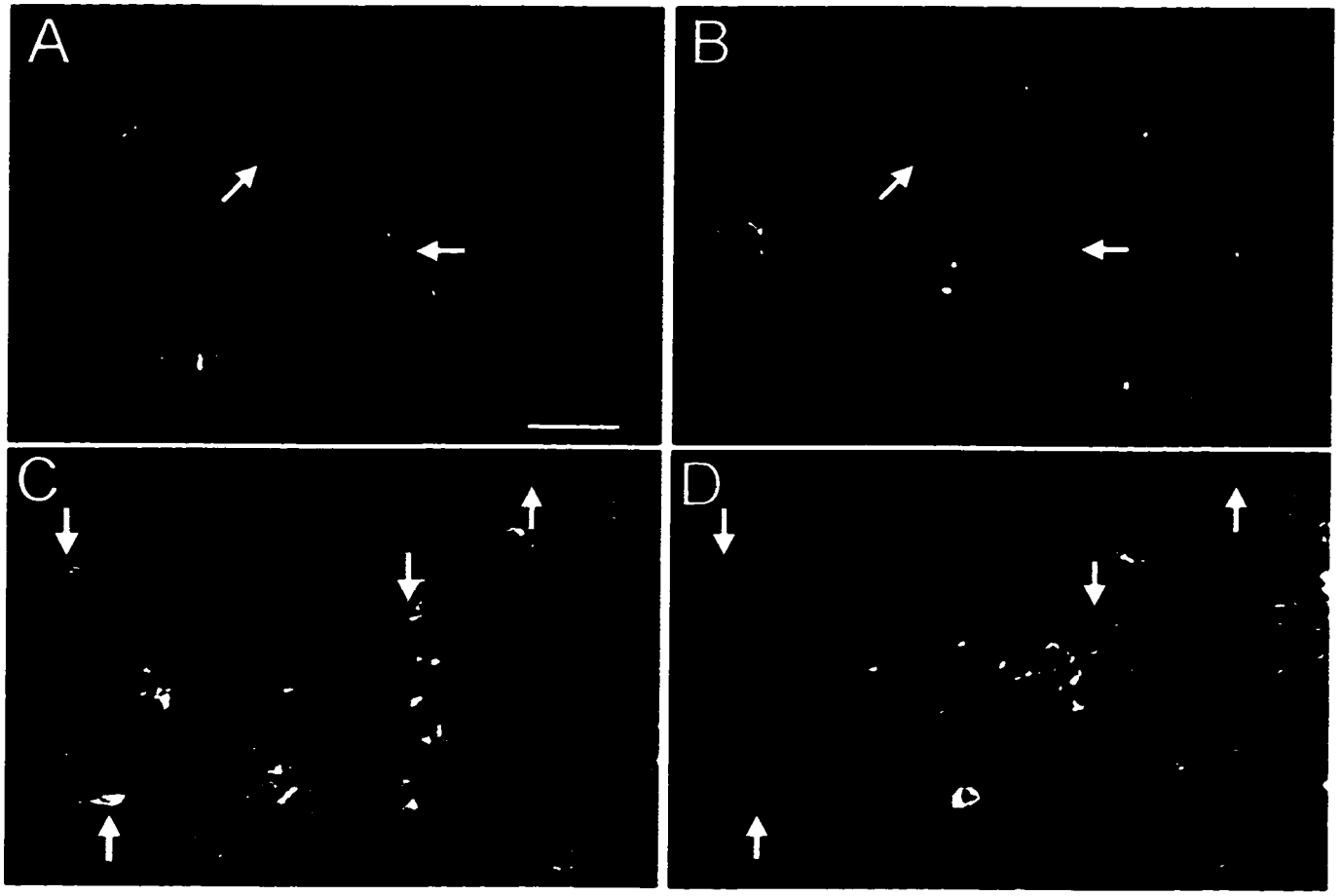
Figure 13. *NG2 immunoreactivity identifies normal and reactive oligodendrocyte progenitors.*

Double immunolabeling for NG2 (A,C) and a microglial cell marker, Mac-1 (B,D) in normal (A,B), and lesioned (C,D) areas of spinal cord. NG2+ cells are not double labeled with Mac-1 in either normal or lesioned white matter. In lesioned white matter Mac-1+ cells (reactive microglia and possibly macrophages) are more intensely immunolabeled and do not have thin processes extended (D). Similarly, NG2+ cells in lesions have less extensive processes and more intense cell body immunolabeling (C), indicative of a reactive phenotype. A,B; same field of lateral white matter. C,D; same field of ventrolateral white matter. Scale bar = 50 μm.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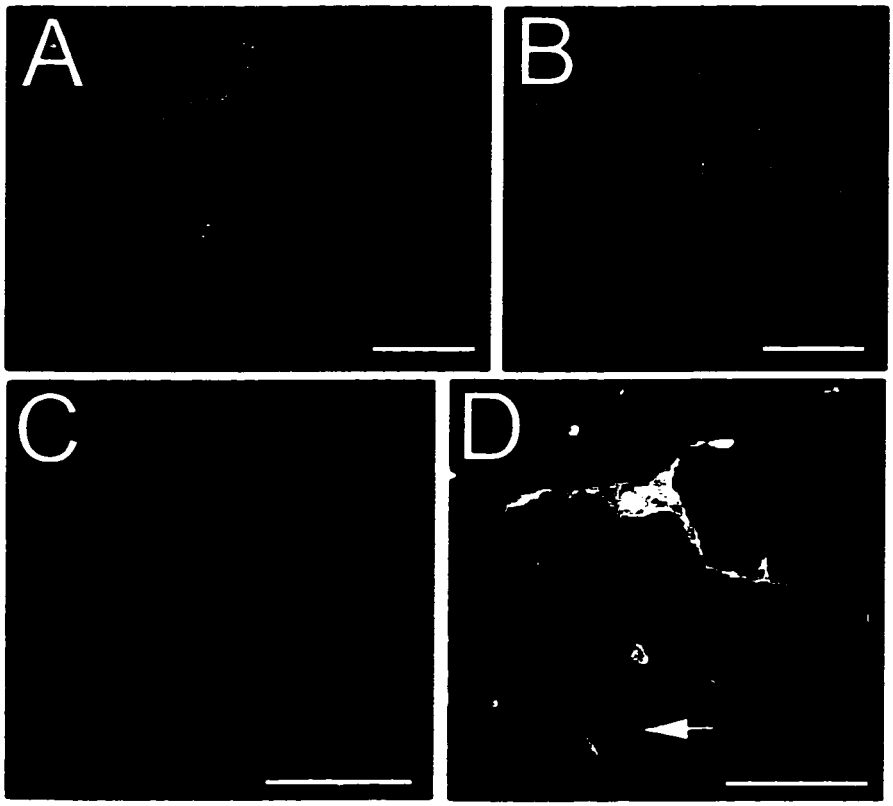
D

Figure 14. *NG2 immunoreactivity does not colocalize with GFAP.* Double immunolabeling for NG2 (A) and an astrocyte marker, GFAP (B) in spinal cord tissue from 4 wpi showing an area of lesion in the superficial dorsal column white matter (top) with normal appearing white matter deeper in the area of the corticospinal tract (A,B). Within and adjacent to the lesion, NG2+ cells (A) are distinct from GFAP+ cells (B). The arrows indicate the location of examples of NG2+ GFAP- cells. To facilitate interpretation of the double immunostaining, (C) shows enlargement of the area of most intense immunoreactivity (arrows correspond to top two arrows in A and B) with NG2 immunoreactivity pseudocolored in red and merged with GFAP immunoreactivity pseudocolored in green. NG2 immunostaining performed by Karen Blinder, and figure prepared by Regina Armstrong. Scale bar = 50 μm.

Figure 15. *Oligodendrocyte progenitors co-express PDGFαR s and FGFRs.*

Multi-label immunofluorescence for established markers for oligodendrocyte progenitors (NG2 in A and B or PDGFαR in C and D) in combination with anti-FGFR, in sections of remyelinating spinal cord (4 wpi). Panels A and B show NG2 immunoreactivity (red) as well as immunoreactivity for FGFRs (green) with double-labeled pixels appearing brown to yellow in each merged image of the digital data sets from single optical sections. The cell in panel A was located in normal-appearing white matter adjacent to a lesion while the cells in panel B were located within a lesion. Panels C and D show single plane optical sections merged and pseudocolored after detection of triple-label immunofluorescence. Panel C shows 2 small cells, located near the gray matter/white matter border at the periphery of a lesion, expressing PDGFαR (red), and FGFRs (green), but not GFAP (blue). Yellow represents colocalization of PDGFαR and FGFRs. Panel D shows an area of deep dorsal column white matter adjacent to a lesion with colocalization of PDGFαR and FGFRs in yellow for a small GFAP negative cell (arrowed), and with colocalization of GFAP (blue), PDGFαR (red), and FGFRs (green) in white for an astrocyte (larger top cell). Scale bar = 6 μm in A,B; and = 12 μm in C,D.


A
B
C
D

Discussion

Normal CNS tissue

In normal mouse tissue, adult oligodendrocyte progenitors express PDGFαR and variable levels of FGFRs. To detect FGFRs, a monoclonal IgM antibody (clone VBS1) was used that binds to FGFR1, and to lesser extent FGFR2 (Venkateswaran *et al.*, 1992). Both of these receptor subtypes bind the bFGF ligand. Colocalization of PDGFαR and FGFRs on adult oligodendrocyte progenitors *in vivo* was not previously reported. These cells may be able to respond to PDGF-AA and/or bFGF, either in normal conditions, or perhaps in response to demyelinating stimulus or injury. Colocalization of PDGFαR and FGFR on adult olidodendrocyte progenitors *in vivo* strengthens the argument that *in vitro* studies that show adult oligodendrocyte progenitors proliferate in response to either PDGF-AA or bFGF, and proliferate at an increased rate in response to both PDGF-AA and bFGF (Wolswijk and Noble, 1992; Engel and Wolswijk, 1996) have relevance to *in vivo* OLCs.

Mature oligodendrocytes expressed FGFRs. Although mature oligodendrocytes proliferate in response to bFGF in culture (Fressinaud *et al.*, 1993), the *in situ* function of bFGF on oligodendrocytes is not clear, since minimal proliferation of these cells is observed. Possibly, bFGF

could act as a survival factor for mature oligodendrocytes and/or could allow mature oligodendrocytes to maintain a degree of plasticity to compensate for normal cell turnover.

In addition to adult oligodendrocyte progenitors, cells in the SVZ also expressed PDGFαR and FGFRs. The SVZ is thought to be a germinal zone for neuronal, astrocyte, and oligodendrocyte precursors that persist into adulthood (Lois and Alvarez-Buylla, 1994; Gritti *et al.*, 1996).

In other regions of the CNS, adult oligodendrocyte progenitors are the only glial cell in gray or white matter that expresses PDGFαR (Nishiyama *et al.*, 1997; Reynolds and Hardy, 1997; Trapp *et al.*, 1997). However, some neurons in the mouse spinal cord displayed a low level of PDGFαR immunoreactivity. Other studies have also reported PDGFαR immunoreactivity, as well as mRNA, in normal mouse spinal cord neurons (Vignais *et al.*, 1995; Oumesmar *et al.*, 1997).

FGFR immunoreactivity was detected with the VBS1 antibody on neurons and astrocytes, in addition to adult oligodendrocyte progenitors. Other studies have examined bFGF receptor subtype distribution in the rodent CNS. They report FGFR1 expression at the mRNA and protein level in neurons (Asai *et al.*, 1993; Yazaki *et al.*, 1994; Gonzales *et al.*, 1995; Belluardo *et al.*, 1997), as well as in astrocytes and oligodendrocytes

(Gonzales *et al.*, 1995). FGFR2 mRNA expression in the rodent CNS has been reported primarily in white matter areas, indicating expression by glial cells (Asai *et al.*, 1993; Yazaki *et al.*, 1994; Belluardo *et al.*, 1997).

Remyelinating CNS tissue

In the local area of CNS injury, NG2+ oligodendrocyte progenitors exhibit a "reactive" phenotype characterized by more intense immunoreactivity and shorter, thicker cell processes (Levine, 1994; Nishiyama *et al.*, 1997). Consistent with these studies, similar morphologic changes and increased immunoreactivity were observed for both NG2+ and PDGFαR+ cells, associated with oligodendrocyte progenitors, distributed within and near demyelinated lesions.

Oligodendrocyte progenitors present in normal mouse tissue and within and near remyelinating lesions express receptors for PDGF-AA (PDGFαR) and bFGF (FGFRs). Our finding that reactive oligodendrocyte progenitors within and near remyelinating lesions exhibit immunoreactivity for receptors for PDGF and FGF suggests the possibility that these cells may be responsive *in vivo* to these ligands acting singly or in combination. *In vitro* analyses of oligodendrocyte progenitors from normal adult rat optic nerve and spinal cord have indicated that PDGF-AA and bFGF,

acting in combination, can increase proliferation and enhance motility to induce a phenotype similar to the oligodendrocyte progenitor in neonatal CNS (Wolswijk and Noble, 1992; Engel and Wolswijk, 1996). Whether PDGF-AA could also serve to enhance survival of oligodendrocyte progenitors of adult rodent CNS, as has been reported for neonatal progenitors and newly formed oligodendrocytes *in vitro* (Barres *et al.*, 1992), has not been determined.

Further, reactive astrocytes also express PDGFαR and FGFRs, and may also be capable of responding to the ligands during remyelination.

The significance of growth factor receptor expression depends upon the presence of the ligands. bFGF immunoreactivity has been detected in microglia and astrocyes in normal tissue in our laboratory (see Redwine *et al.*, 1997), and bFGF expression in neurons and astrocytes has been reported by others (Gonzales *et al.*, 1995). Work characterizing bFGF ligand expression in normal and remyelinating tissue will continue by others in the lab.

The PDGF-AA ligand has been detected in normal and remyelinating tissue. These data are presented in the next chapter. A cellular response associated with PDGF-AA, specifically cell proliferation, and the expression of the PDGFαR are also examined in the next chapter.

In addition to identification of oligodendrocyte progenitors within and near lesions, and the identification of growth factor receptor expression on oligodendrocytes and astrocytes, a complex cellular response within and near remyelinating lesions was reported in this chapter. Increased immunoreactivity for both NG2+ and PDGFαR+ cells associated with reactive oligodendrocyte progenitors was observed within and near demyelinated lesions. Immunoreactivity for microglia and astrocyte markers was also increased within and near lesions. Preliminary data were mentioned identifying B lymphocytes within and near lesions as well. This cellular response is similar, but not identical, to the cellular response observed in some MS plaques.

Demyelinated plaques seen in MS are heterogeneous (Prineas *et al.*, 1992; Lucchinetti *et al.*, 1996). However, increased levels of astrocytes (Newcombe *et al.*, 1986; Prineas *et al.*, 1990), microglia (Gay *et al.*, 1997) and B-lymphocytes (Nyland *et al.*, 1982) can be found in some MS plaques. As discussed in the Introduction, oligodendrocyte progenitors have been identified in human brain *in vivo* and in glial cultures derived from human brains. One recent study reported oligodendrocyte progenitors within MS plaques labeled with progenitor O4, but not with galactocerebroside, GFAP, or vimentin (Wolswijk, 1998). These

progenitors had an oval or round cell body with fine processes. However, the presence of adult oligodendrocyte progenitor cells within or near MS lesions is not well characterized. Specific markers for rodent oligodendrocyte progenitors, such as NG2 and PDGFαR, have not worked well in available human MS tissue (Wolswijk, 1998). Therefore, it is unknown whether the remyelination seen in some MS plaques is associated with adult oligodendrocyte progenitors similar to those characterized in the rodent CNS, or with mature oligodendrocytes.

Further examination of the complex cellular responses (*i.e.*, characterization of cell phenotypes) in the MHV-A59 model of successful remyelination, and further examination of the complex cellular response in MS, may lead to an understanding of the abortive, incomplete remyelination seen in human MS patients, and may lead to the development of therapies to promote a more complete remyelination.

Chapter 5

PDGF and Cell Proliferation During Remyelination

The data presented in Chapter 3 characterized a time point corresponding to early remyelination. The data presented in Chapter 4 demonstrated the presence of adult oligodendrocyte progenitors within and near remyelinating lesions, and showed that these cells express receptors for PDGF-AA and bFGF. Data are presented in this chapter that associate *in vivo* proliferation of adult oligodendrocyte progenitors with local increases in PDGF-A and the PDGFαR.

Non-radioactive in situ hybridization detected local expression of PDGF-A ligand and receptor.

Non-radioactive *in situ* hybridization using digoxigenin (DIG) incorporation into ribonucleotide probes was a sensitive technique that detected PDGF-A and PDGFαR steady state mRNA, and detected differences between normal and remyelinating spinal cord. Cell morphology was clearly visible following detection of mRNA with the bluish/purplish colored precipitate. Figure 16 describes the preparation of ribonucleotide probes.

PDGF-A ligand during remyelination

An increased number of cells express PDGF A during remyelination.

In situ hybridization and immunostaining were performed to determine whether PDGF-A ligand is potentially available to activate the PDGFαR *in vivo*. In sections from both normal and 4 wpi lesioned spinal cords, the *in situ* hybridization analysis demonstrated that PDGF-A mRNA transcripts were present at a low level in neurons throughout the gray matter with much more intense signal in some smaller non-neuronal cells in the gray matter and white matter (Figure 17). The neuronal signal did not appear to vary between normal and lesioned sections. In contrast, the number of intensely labeled cells per section from PBS-injected mice was 5.6 (SE = 1. 2; n= 8 cervical sections; 2-3 sections per mouse from each of 3 mice) with an increase to 16.3 cells per section from lesioned mice (SE = 1.4; n = 9 cervical sections; 1-3 sections per mouse from each of 5 mice). Sections taken from mice sacrificed at 3 wpi appeared similar to those at 4 wpi (not shown). This approximately 2.9 fold increase represents a significant increase in the number of cells expressing PDGF-A mRNA in 4 wpi lesioned spinal cord sections compared to normal tissue ($p < 0.0001$ when compared by Student's unpaired t-test).

PDGF-A immunoreactivity in normal adult mouse spinal cord is

Figure 16. *Construction of PDGF-A (panels A-D) and PDGFαR (panels E-H) DIG-labeled RNA probes used for in situ hybridization.*

Schematic diagrams are shown of cDNA templates for PDGF-A (A, kindly provided by Mark Mercola), and for PDGFαR (E, kindly provided by Bill Richardson). The bands in panels (B,C,F,G) are ethidium bromide-labeled DNA fragments run on 1.2% agarose gels. Arrows pointing to lane 2 of (B) and (F) indicate appropriate sizes of PDGF-A (B) and PDGFαR (F) inserts. Arrows pointing to bands in lane 2 of panels (C) and (G) indicate linearized plasmids in preparation for *in vitro* RNA transcription. The PDGF-A construct was linearized with Sac1 for transcription of antisense RNA using T7 polymerase, and the PDGFαR construct was linearized with HindIII for transcription of antisense RNA (lane 2, panel G) using T7 polymerase, or with EcoR1 for sense RNA using SP6 polymerase (not shown). Panels (D) and (H) are dot blots verifying DIG-UTP incorporation for PDGF-A antisense RNA (lane 2, panel D), PDGFαR antisense RNA (lane 2, panel H), and PDGFαR sense RNA (lane 3, panel H). Pre-DIG-labeled RNA controls are shown (lane 1, panels D,H), as well as DIG-labeled RNA from beta actin DNA template (lane 3, panel D; lane 4, panel H), and *in vitro* transcription without a DNA template as a negative control (lane 5, panel H).

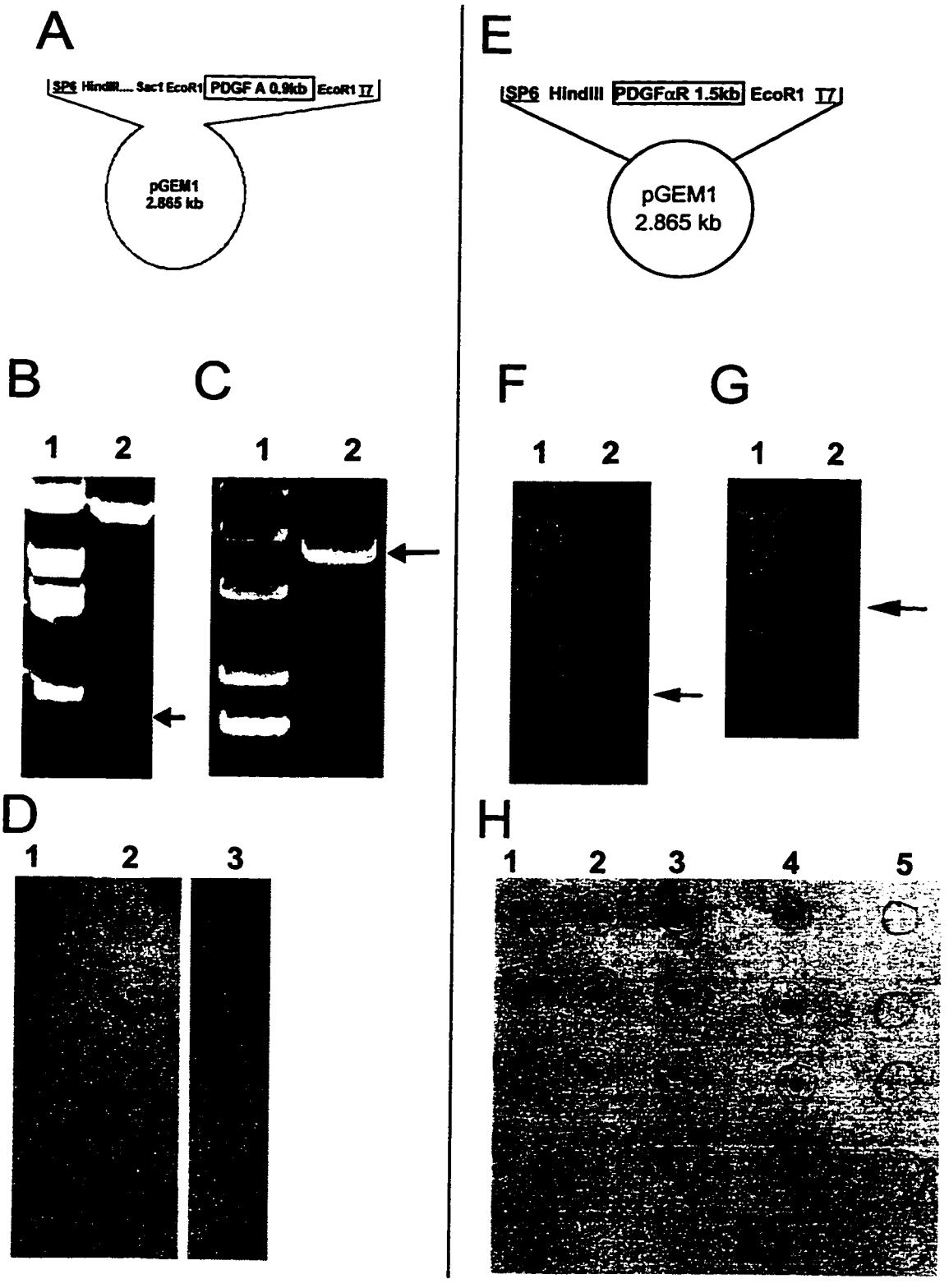

A
SP6 HindIII.... Sac1 EcoR1 PDGF A 0.9kb EcoR1 T7
pGEM1
2.865 kb
E
SP6 HindIII PDGFαR 1.5kb EcoR1 T7
pGEM1
2.865 kb
B
C
F
G
1 2
1 2
1 2
1 2
D
1 2 3
H
1 2 3 4 5

Figure 17. *PDGF-A mRNA transcripts in normal and lesioned spinal cord.*

In situ hybridization for PDGF-A mRNA transcripts in sections from PBS-injected mice (A) or 4 wpi MHV-injected mice (B,C,D,E,F). A subpopulation of glial-like cells with intense signal is present but infrequent in normal tissue (A, examples at black arrows) and detected more frequently in sections from lesioned mice (B,C,D,E,F; examples at black arrows), either at the edge of white matter lesions (B), or within vacuolated white matter lesions (D, examples at black arrows). Neurons typically have a low level or undetectable level of signal in normal (A) and lesioned (B) sections. However, panel C shows detectable level of PDGF-A expression that is visible in some sections in large ventral motor neurons (examples at white arrows in C), in addition to PDGF-A mRNA detectable in smaller glial cells (black arrow in C). Panels E and F are examples of large glial cells expressing PDGF-A mRNA near the edge of white matter lesions. The morphology of these cells is similar to large, reactive GFAP+ astrocytes seen in Figure 18 and Figure 20. Scale bar in B = 200 μm for A,B; scale bar in D = 50 μm for C,D,E.

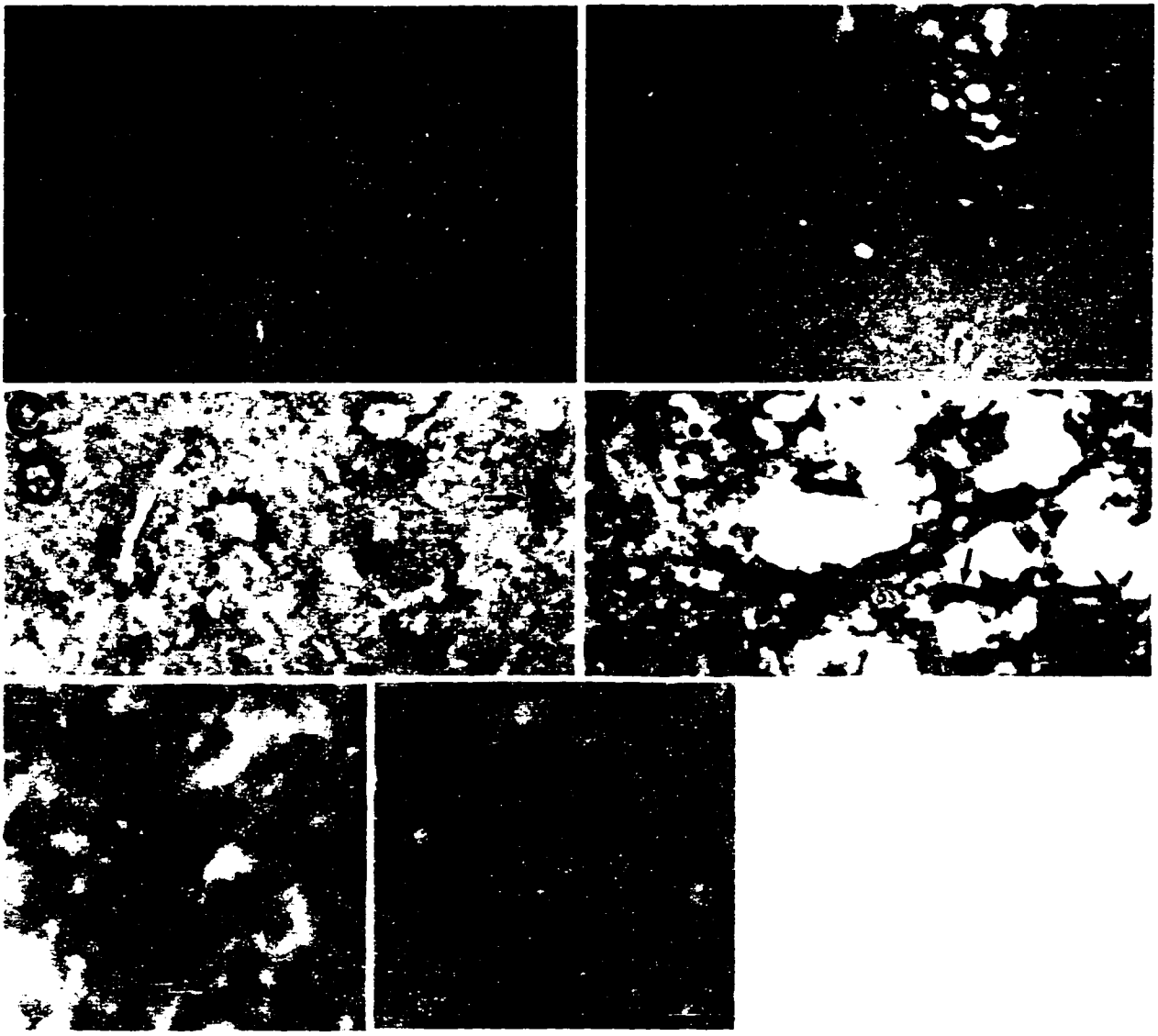
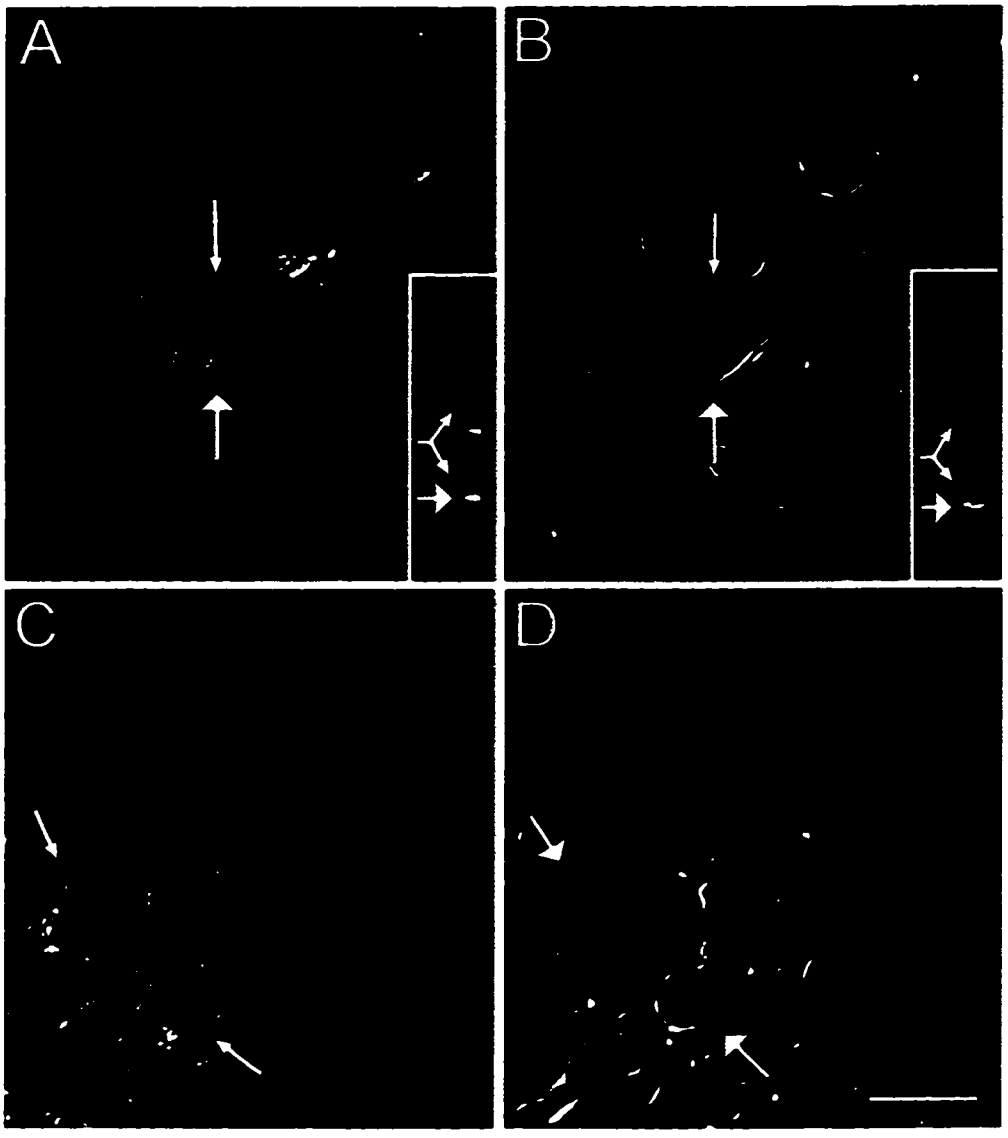


Figure 18. *PDGF immunoreactivity in normal and lesioned white matter.*

Double-label immunofluorescence for PDGF-A (A,C) and GFAP (B,D) is shown as 3-D reconstructions of 10 (A,C) or 20 (B,D) optical planes (each 0.25 μm) acquired and restored with the CELLscan system. Control (A,B) and lesioned (C,D) ventral white matter areas are shown in the xy dimension, as in a transverse section of spinal cord. In control tissue, PDGF-A ligand immunoreactivity is detected at low levels within axons (structure crossing field diagonally in A) and within some GFAP + astrocyte processes (large arrow in A and B) that are radially oriented along axons. The insets in (A) and (B) each show the yz dimension of a 3-D reconstruction of an optical plane taken from between the large and small arrows (across the axon diameter) which shows that PDGF-A immunoreactivity is within the axon (small diverging arrows) and within the astrocyte process (large arrows). In normal spinal cord, PDGF-A immunoreactivity is detected in very few astrocytes (1 PDGF-A immunoreactive astrocyte in the field shown in panels A and B). In remyelinating lesions, there are many reactive astrocytes with detectable levels of PDGF-A immunoreactivity (3 PDGF-A immunoreactive astrocytes in the field shown in panels C and D). Scale bar = 10 μm.


A
B
C
D

present at low levels in neurons, axons, and in some astrocytes oriented radially along axons (Figure 18). Within remyelinating lesions, at 4 wpi, PDGF-A immunoreactivity appeared to be locally increased and primarily associated with reactive GFAP + astrocytes (Figure 18). Our lab has previously demonstrated that bFGF is found at low levels in distributed astrocytes and microglia in normal adult mouse white matter (Redwine *et al.*, 1997). Therefore, within and near white matter lesions, PDGF-A and bFGF may be locally available to potentially regulate oligodendrocyte progenitors expressing PDGFαR and FGFRs.

PDGFαR and adult oligodendrocyte progenitors during remyelination

The number of small, elongated cells that expressed PDGFα receptor mRNA was increased during remyelination.

To determine if the number of PDGFαR expressing cells is increased during remyelination, *in situ* hybridization was performed. In control cervical spinal cord 4 weeks after the PBS injection, small cells expressing PDGFαR mRNA are distributed throughout the white matter, and sparsely throughout the gray matter (Figure 19c). These cells have a small elongated morphology that resembles white matter oligodendrocyte progenitors (Chapter 4, Figures 13,14,15). In regions such as the dorsal

horn gray matter these cells may appear similar to small neurons also expressing PDGFαR mRNA, although neurons have a lower level of expression of PDGFαR relative to oligodendrocyte progenitors (Oumesmar *et al.*, 1997). Large ventral motor neurons did not express detectable levels of PDGFαR mRNA under the present conditions. In MHV-A59 injected mice at 4 wpi, there is a 2-fold increase in the number of cells that express PDGFαR mRNA in lesioned cervical spinal cord compared to control tissue sections (Table II). The increase of PDGFαR mRNA expressing cells occurred primarily within and near lesioned areas of white matter (Figure 19). This pattern matches the pattern seen of PDGFαR immunoreactive oligodendrocyte progenitors (*i.e.*, increased within or near the edge of the lesion).

Proliferating cells during remyelination

An in vivo BrdU pulse detected proliferating cells.

MHV-infected mice and PBS-injected control mice were injected i.p. with 200 mg/kg BrdU at 4 and 2 hours prior to sacrifice at 4 wpi. This pulse protocol allowed sufficient nuclear BrdU labeling while minimizing the time for potential cell differentiation or alteration in expression of growth factor receptors.

Some PDGFαR mRNA expressing oligodendrocyte progenitors were proliferating during remyelination.

To determine whether oligodendrocyte progenitors in and near lesions actively proliferate at this stage and whether PDGF-A is a potential mitogen for oligodendrocyte lineage cells contributing to remyelination, we examined *in vivo* BrdU incorporation by cells expressing the PDGFαR. In this mouse spinal cord tissue a protocol for the simultaneous detection of BrdU incorporation and immunostaining for the oligodendrocyte progenitor markers, NG2 and PDGFαR, could not be worked out (unpublished observation). Therefore, we used *in situ* hybridization to identify oligodendrocyte lineage cells that were BrdU+. Based upon previous studies (Pringle *et al.*, 1992; Oumesmar *et al.*, 1997) and immunostaining data presented in Chapter 4, we expect that small, elongated cells expressing PDGFαR mRNA correspond with oligodendrocyte progenitors (Figure 19).

There was a 14.5-fold increase in the number of small elongated cells that expressed PDGFαR mRNA and exhibited nuclear BrdU immunoreactivity in remyelinating spinal cord compared to control tissue (Table II, Figure 19). This phenotype is consistent with oligodendrocyte progenitors and is distinct from the larger, reactive PDGFαR+ astrocytes (Chapter 4, Figure 15). The number of PDGFαR mRNA+/BrdU+ cells

identified per section is significantly increased in the lesioned tissue ($p < .0005$, determined by an unpaired Student's t-test) even though the total number of labeled cells is small due to the brief 4 hour BrdU terminal labeling period. In this animal model, remyelination and presumably repopulation of the lesion soccurs over a period of days or even weeks. Therefore, if this rate is maintained, an increase of presumed oligodendrocyte progenitors could result in a substantial population of oligodendrocyte lineage cells.

The number of proliferating cells increased during remyelination.

In sections of cervical spinal cord from MHV-A59 injected mice at 4 wpi, the number of BrdU+ cells is increased 30-fold compared to sections from PBS-injected control mice (Table II). In separate experiments with BrdU/GFAP double label (not represented in Table II), many of the BrdU+ cells, especially those with large, oval nuclei, were GFAP+ (Figure 20; see also Godfraind *et al.*, 1989 and Armstrong *et al.*, 1990). Round cells expressing PLP mRNA represent oligodendrocytes. These cells did not incorporate BrdU (Figure 21).

The identification of small, elongated PDGFαR expressing adult oligodendrocyte precursors incorporating BrdU, in combination with the

Figure 19. *PDGFαR mRNA in cells that proliferate in remyelinating spinal cord.*

In situ hybridization was performed on 15 μm cervical spinal cord transverse sections to detect PDGFαR mRNA followed by immunodetection of BrdU incorporated during a 4 hr *in vivo* pulse. PDGFαR mRNA antisense signal (black arrows) combined with BrdU immunodetection (white arrows) is shown in spinal cord with a ventrolateral lesion at 4 wpi (A) compared to PBS-injected control tissue (C). PDGFαR sense probe with BrdU incorporation in remyelinating spinal cord is shown as a control for hybridization specificity (D). Higher magnification of PDGFαR mRNA antisense and BrdU labeling in remyelinating lesions (B) shows colocalization of PDGFαR mRNA and BrdU incorporation (B, arrow with black and white). E-G show additional high magnification examples of PDGFαR mRNA+ cells from similar lesions. Panel (E) shows two PDGFαR mRNA+ small elongated cells that did not incorporate BrdU. Two similar examples of PDGFαR mRNA+ cells with nuclear BrdU immunolabel are shown in panels (F) and (G). Scale bar = 200 μm for A,C,D, and 50 μm for B, E-G.

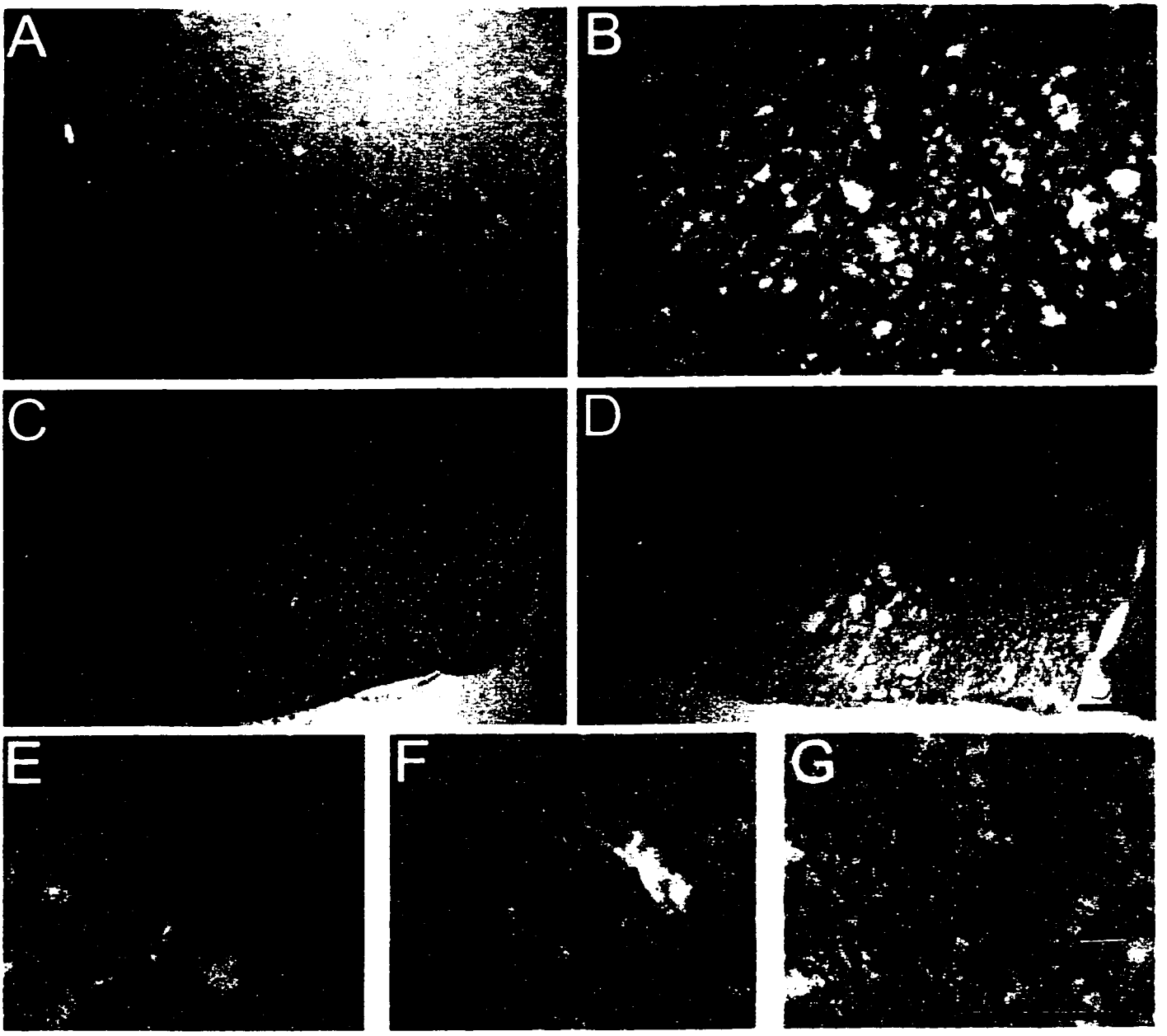

A
B
C
D
E
F
G

Figure 20. *Astrocyte proliferation in response to demyelination.* Double immunolabeling of mouse spinal cord sections (4 wpi) to detect GFAP (green) in combination BrdU (red) incorporated during a 4 hr *in vivo* pulse. In the normal appearing white matter (A), rare BrdU+ cells could be found which had GFAP immunoreactivity. Around the edge of a lesion (B), numerous large reactive astrocytes with strong GFAP immunoreactivity are present toward the center of the lesion (top of panel B) and one cell (center of panel B) has GFAP immunoreactivity in the cytoplasm with BrdU immunolabeling in the nucleus. Several small cells (bottom of panel B) located further from the lesion center have BrdU immunolabeled nuclei, but are not immunolabeled for GFAP. Immunofluorescence was performed by Tuan Lee and Photography by Regina Armstrong. Scale bar = 50 μm.

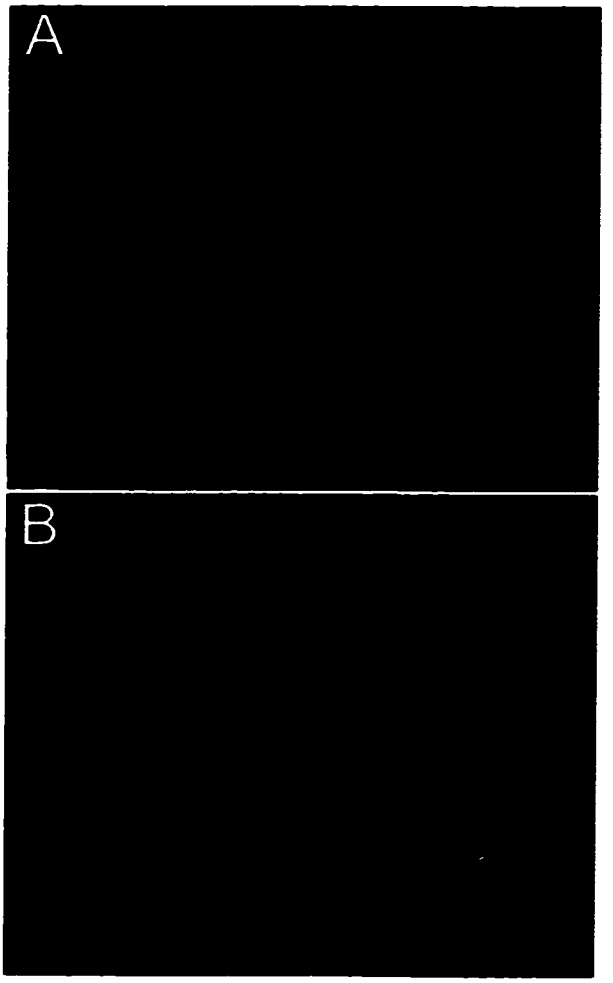

A
B

Table II. Counts of small elongated PDGFαR mRNA and BrdU Labeled Cells

type of cellular label	PBS (n=2) (cells/section) mean	[S.E.]	MHV (n=5) (cells/section) mean	[S.E.]	P value	fold increase
PDGFαR mRNA	49	[6.6]	98*	[6.8]	<.0005	2
BrdU	3	[0.5]	82*	[15.8]	<.0005	30
PDGFαR mRNA/ BrdU double label	0.4	[0.2]	6.4*	[0.7]	<.0005	15

In situ hybridization on 15 μm cervical spinal cord transverse sections targeting PDGFαR mRNA was followed by immunostaining for BrdU (incorporated during proliferation following a 4 hr BrdU pulse). Cells expressing PDGFαR mRNA, immunolabeled for BrdU, or both were counted, and data are presented as the average number of cells per section (4-5 sections per 2 PBS mice; 2-3 sections per 5 MHV mice). Asterisks represent statistical significance. P values were obtained using an unpaired Student's t-test. S.E.= standard error.

Figure 21. *Proliferating cells do not have detectable PLP mRNA.* *In situ* hybridization was performed on 15 μm cervical spinal cord transverse sections to detect PLP mRNA followed by immunodetection of BrdU incorporated during a 4 hr *in vivo* pulse. A reduced number of PLP mRNA expressing cells is detected in a ventral lesion at 4 wpi (lower left in B) compared to adjacent non-lesioned white matter (B) or PBS-injected control tissue (A). BrdU labeled cells (white arrows) within and near the edge of lesions did not have signal for PLP mRNA in contrast to many cells with an oligodendrocyte morphology (black arrows) (C). Scale bar = 200 μm in A-B, and 50 μm in C.


A
B

lack of BrdU incorporation observed for oligodendrocytes, identified by PLP mRNA expression (Figure 21), indicate that PDGFαR+ oligodendrocyte progenitors are a significant source of newly generated oligodendrocyte lineage cells during remyelination.

Discussion

Lesions at 4 wpi have dramatically reduced PLP mRNA expression (Figure 21), while exhibiting increased immunoreactivity for oligodendrocyte progenitors (Chapter 4, Figures 12,13,14). This data, in combination with behavioral and histopathologic analysis (Chapter 3), indicate that 4 wpi corresponds with a stage of early remyelination and adult oligodendrocyte progenitor repopulation of lesions. At this time point, there are increases in 1) the number of cells expressing PDGF-A mRNA, 2) the number of cells expressing PDGFαR mRNA, 3) the number of small, elongated oligodendrocyte progenitors expressing PDGFαR mRNA and proliferating, and 4) the number of other proliferating cells measured by BrdU incorporation.

Local changes in the availability of PDGF ligand may modulate oligodendrocyte progenitor responses in and around lesions. *In situ* hybridization analysis demonstrated PDGF-A chain mRNA in neurons which was not detectable in well-developed GFAP+ astrocytes in myelinated corpus collosum of normal rats (Ellison *et al.*, 1996). In the spinal cord white matter of control adult mice in the present study, we show that immunoreactivity for the PDGF-A ligand was present at low levels mainly in neuron cell bodies and axonal processes, but could also be

found in astrocytes oriented radially along the axons. In remyelinating lesions, an increased number of cells expressed relatively high levels of PDGF-A mRNA transcripts, while immunoreactivity for PDGF-A was locally increased in GFAP+ reactive astrocytes. Since PDGF acts as a dimer, immunostaining for the PDGF-A ligand may detect not only the PDGF-AA homodimer, but also the PDGF-AB heterodimer, both of which can act through the PDGFαR of cultured neonatal oligodendrocyte progenitors, which can additionally be activated by PDGF-BB (Pringle *et al.*, 1989). Reactive astrocytes may synthesize and secrete PDGF to locally increase its availability in lesions. This hypothesis is derived from the observation that cultured neonatal rat astrocytes, as well as neurons, synthesize and secrete PDGF (Richardson *et al.*, 1988; Dutly and Schwab, 1991). Since PDGF-A, either alone or in combination with bFGF, can induce proliferation of adult oligodendrocyte progenitors *in vitro* (Engel and Wolswijk, 1996), PDGF may be inducing adult oligodendrocyte progenitors to proliferate *in vivo* in response to demyelination.

Cell proliferation not associated with PDGFαR expression was evident. Other growth factors may be involved in remyelination in this model. These could include IGF-1, bFGF, or PDGF-AA plus glial growth factor-2 (GGF-2) acting together (Shi *et al.*, 1998).

During the very early stage of remyelination examined in this study, repopulation of the lesion by OLCs appeared to be ongoing. Cell proliferation was increased within and near remyelinating lesions compared to non-demyelinated or control white matter. Proliferating BrdU+ cells were localized within and near lesion areas and were most frequent along the edge of a remyelinating lesion. This distribution of BrdU+ cells is consistent with recruitment of remyelinating cells from the local periphery (Franklin *et al.*, 1997). As expected from previous studies of MHV-A59 lesioned tissue (Godfraind *et al.*, 1989; Armstrong *et al.*, 1990b), a large component of the proliferating cell population appeared to be astrocytes with large, oval nuclei. Distinct from astrocytes were the small, elongated oligodendrocyte progenitor cells, PDGFαR transcript positive. This progenitor population exhibited a significant increase in the proportion of cells that incorporated BrdU. In contrast, oligodendrocytes were identified by expression of PLP mRNA transcripts and determined to be a relatively quiescent population which generally did not incorporate BrdU. Our data reflect BrdU-incorporation during a narrow 4 hour window during the remyelination process. This study was designed with a restrictive time interval in order to locate and identify proliferating OLCs while minimizing the potential for differentiation or other phenotypic

changes. However, OLC proliferation is likely to occur over a much longer period of time, because as early as one wpi OLC proliferation was demonstrated in the MHV-A59 model (Godfraind *et al.*, 1989). Additionally, in other models OLC proliferation was shown to occur over days to weeks (Herndon *et al.*, 1977; Ludwin, 1979). The BrdU+ cells identified as PDGFαR+ oligodendrocyte progenitors in the present study are likely to give rise to remyelinating oligodendrocytes since a previous study of remyelination after MHV-A59 infection demonstrated that ^{3}H-thymidine administered at 4 wpi was detectable in oligodendrocytes in mice sacrificed 5 months later, when remyelination was completed (Godfraind *et al.*, 1989).

These studies do not preclude the potential of surviving mature oligodendrocytes to contribute to remyelination by proliferating, which has been suggested (Aranella and Herndon, 1984; Ludwin and Baker, 1988; Wood and Bunge, 1991). However, the phenotype of OLCs at the time of proliferation (either immature oligodendrocyte progenitor or mature oligodendrocyte) was not clear in these previous studies. Proliferating mature oligodendrocytes were not detected in this study using PLP mRNA as a marker of mature myelinating oligodendrocytes. The present findings are consistent with recent demonstrations that oligodendrocyte lineage cells

proliferate prior to remyelination and that mature oligodendrocytes are post-mitotic (Keirstead and Blakemore, 1997; Carroll *et al.*, 1998). In addition, our findings are consistent with many studies which have concluded that proliferation of a progenitor population serves as a source of remyelinating oligodendrocytes (Ludwin, 1979; Godfraind *et al.*, 1989; Prayoonwiwat and Rodriguez, 1993; Gensert and Goldman, 1997; Carroll *et al.*, 1998; Keirstead *et al.*, 1998). The continuity from an endogenous cycling progenitor to a remyelinating cell has been demonstrated by retroviral labeling prior to lysolecithin-induced demyelination and subsequently observing remyelinating oligodendrocytes (Gensert and Goldman, 1997). The present studies support a lack of proliferation of mature oligodendrocytes and extend previous reports of progenitor proliferation by demonstrating the expression of growth factor receptors and ligands that could serve as potential mitogens in this response. As progenitor cells in adult CNS become better characterized, it will be important to determine whether there are subpopulations of precursor cells that differentially express molecules, such as PDGFαR and NG2, and therefore may exhibit distinct responses to demyelination.

The increased immunoreactivity for markers of oligodendrocyte progenitors, NG2 and PDGFαR, and the increased proliferation of

PDGFαR+ cells observed during this early stage of remyelination in the present study indicate a progenitor response associated with repopulation of the demyelinated lesions. Similarly, Keirstead *et al.* (1998) demonstrated locally increased cell number and proliferation of NG2 + cells in response to demyelination which decreased with the progress of remyelination. However, in interpreting the present data, additional functions of oligodendrocyte progenitors should be considered, as well as the possibility that additional cells could be driven to express PDGFαR and/or NG2 in pathological conditions. The frequency of NG2 + cells and the extent of process formation in normal rodent CNS is remarkable, indicative of the possibility that these cells may serve as more than just a reserve of precursor cells persisting in the adult CNS (Levine, 1994; Nishiyama *et al.*, 1997). The processes of NG2+ oligodendrocyte progenitors often are adjacent to microglia suggesting a functional relationship between these two cell types (Nishiyama *et al.*, 1997). The plasticity response of the NG2 + population associated with CNS lesions is also indicative of possible functions as "reactive" cell types. Nishiyama *et al.* (1997) observed an increased number of reactive NG2+ cells during the acute inflammatory phase of experimental allergic encephalomyelitis and Levine *et al.* (1994) reported reactive changes and proliferation of NG2+ cells following stab

injury. Therefore, NG2 + cells may respond to signals in the pathological environment that may be more general than just myelin damage. This observation would be consistent with effects mediated through local changes in growth factors and/or localized alteration of neuro-glial interactions.

The data presented and discussed in Chapter 4 demonstrated an increased adult oligodendrocyte progenitor response associated with remyelinating lesions. Also within remyelinating lesions, OLCs were shown to express of PDGFαR and FGFRs (adult oligodendrocyte progenitors) or FGFRs (mature oligodendrocytes), and astrocytes were shown to express both PDGFαR and FGFRs. Data was presented in this chapter that demonstrated significant increases in PDGFαR and PDGF-A expression within and near remyelinating lesions, and a significant increase in adult oligodendrocyte progenitor proliferation associated with PDGFαR mRNA expression *in vivo*. Therefore, an OLC proliferative or repopulative response associated with PDGF during remyelination has been shown.

Determining the *in vivo* role of growth factors, acting singly or in combination, to bring about successful remyelination will be extremely important. In the MHV-A59 model, our findings indicate that the

oligodendrocyte progenitor is likely to be a significant source of newly generated oligodendrocyte lineage cells and we have presented data to justify further examination of the *in vivo* roles of PDGF and FGF in lesion repopulation. These and other growth factors interacting with cell adhesion molecules and/or matrix molecules would be expected to mediate oligodendrocyte progenitor differentiation into mature oligodendrocytes and provide the appropriate signals for subsequent myelination of denuded axons. The present study and several others (Armstrong *et al.*, 1990b; Levine *et al.*, 1994; Nishiyama *et al.*, 1997; Webster, 1997) demonstrate that adult oligodendrocyte lineage cells exhibit phenotypic changes in pathologic CNS, which should be taken into consideration as potentially altering the responses of oligodendrocyte progenitors and oligodendrocytes in a lesion environment as compared to normal adult CNS. As progress is made toward abrogating or attenuating the pathogenic processes in demyelinating diseases, such as multiple sclerosis, our further understanding of these growth factor interactions could possibly guide the design of strategies to promote remyelination in human demyelinating disease.

Concluding Remarks

This study examined growth factor and growth factor receptor expression associated with oligodendrocyte repopulation and remyelination in a mouse model of transient demyelination induced by i.c. injection of MHV-A59.

This study extended previous characterizations of the MHV-A59 model by developing a motor test to monitor motor impairment and motor function recovery. The motor test provided objective criteria for identifying neurologically impaired mice, and identified a time course of motor impairment, associated with demyelination, followed by gradually improved motor function, associated with remyelination. Prior to this study, there were no clear criteria for neurologic impairment in this model, and there was no method of documentation for motor function recovery. Once a clear stage of remyelination was identified by histology and by motor function improvement (4 wpi), OLCs within and near lesions, expression of growth factor and growth factor receptors, as well as growth factor receptor expression associated with OLC repopulation and remyelination were examined.

In accordance with other studies, a population of adult oligodendrocyte progenitors, in addition to the population of mature myelin-producing oligodendrocytes, was identified in normal adult mouse

spinal cord. Adult oligodendrocyte progenitors expressed receptors for growth factors known to induce proliferation *in vitro*, specifically PDGF-AA and bFGF. Mature oligodendrocytes expressed FGFRs, suggesting they may also be able to respond to bFGF *in vivo*.

During remyelination, immunoreactivity for markers specific for adult oligodendrocyte progenitors was increased within and near remyelinating lesions. Adult oligodendrocyte progenitors likely provide a significant source of remyelinating cells. This population of cells had a reactive morphology, as characterized by shorter, thicker processes. These progenitors expressed growth factor receptors for PDGF-AA and bFGF, as was observed in control tissue. Reactive astrocytes also expressed receptors for PDGF-AA and bFGF during remyelination.

PDGF ligand and receptor are locally increased in remyelinating mouse spinal cord. More specifically, the number of cells expressing either the PDGF-AA ligand or the PDGFαR is increased during remyelination compared to controls. In addition, there is an increase in proliferating adult oligodendrocyte progenitors that express the PDGFαR during remyelination compared to controls. These data provide evidence that the mitogenic effect of PDGF on oligodendrocyte progenitors that is well-characterized *in vitro* may also occur *in vivo*, and induce repopulation

of demyelinated lesions.

These findings provide a stepping stone for continuing studies to further confirm that PDGF and bFGF promote OLC-mediated remyelination. Peripheral administration of PDGF may reduce lesion severity, as seen with IGF-1 administration. The extent or time course of remyelination may be altered in MHV-A59 susceptible strains of mutant, transgenic, or knockout mice that over-express or under-express growth factor ligands or receptors. Potential differences among these mice strains compared to normal mice may be detected at the behavioral and/or molecular level. Peripheral administration of growth factor agonists or antagonists may alter the course or extent of remyelination in normal mice following an MHV-A59 injection, providing additional evidence of growth factor involvement in remyelination. Antagonists are available that block PDGF and bFGF receptor activation (Dahring *et al.*, 1997), or block PDGF and epidermal growth factor, but not bFGF-induced proliferation (Williams *et al.*, 1997). Peripheral administration of the PDGF antagonist trapidil has been reported to reduce the extent of remyelination following lysolecithin (McKay *et al.*, 1997). Use of pharmacologic agents to interfere with or enhance PDGF and/or bFGF activity in this model may extend current knowledge of *in vivo* involvement of growth factors during

remyelination.

Ultimately, therapies specifically targeted to OLCs may be developed to halt deteriorating neurologic function seen in MS, and perhaps improve neurologic function in patients with MS or other demyelinating diseases.

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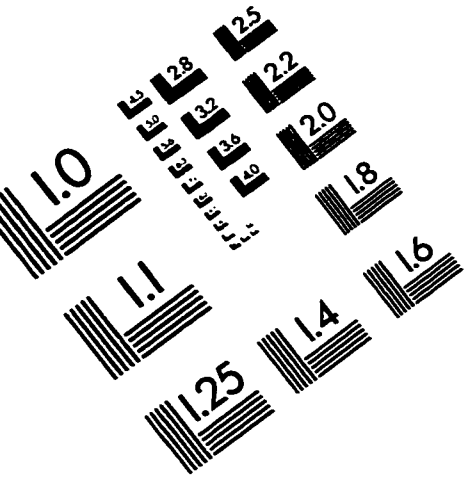
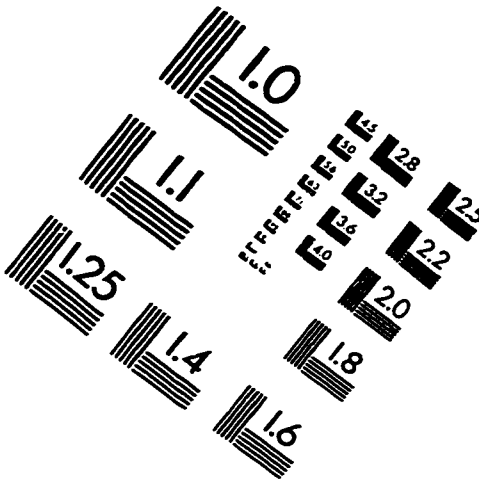
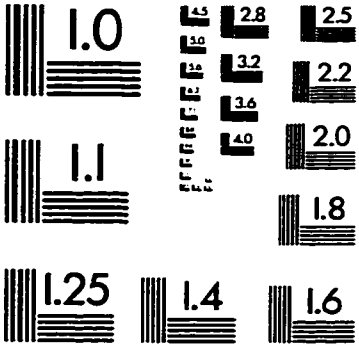
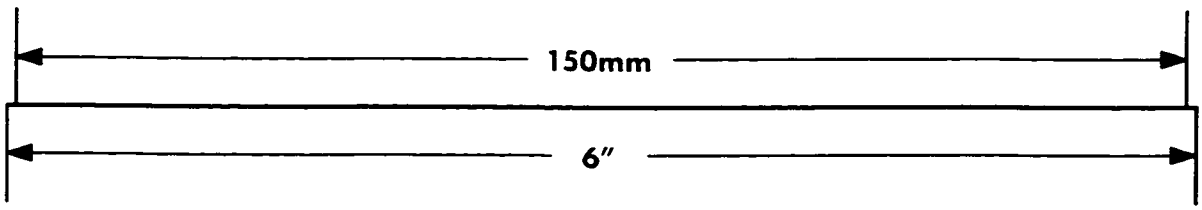
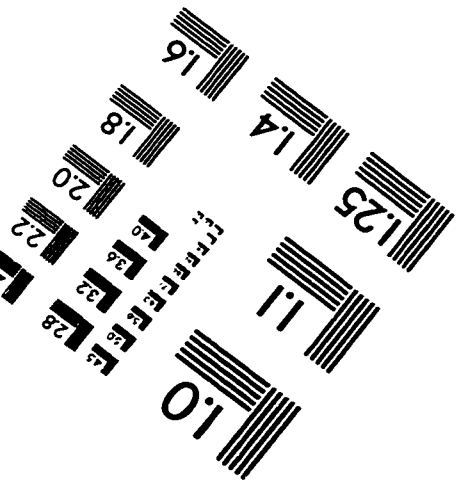
IMAGE EVALUATION TEST TARGET (QA-3)

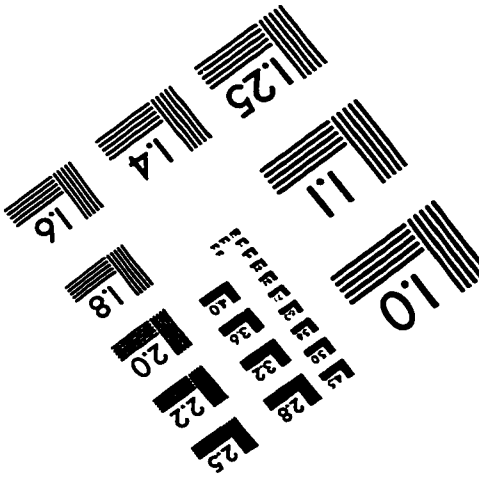












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