

# In Vitro Analysis of the Oligodendrocyte Lineage in Mice during Demyelination and Remyelination

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**Abstract.** A demyelinating disease induced in C57Bl/6N mice by intracranial injection of a coronavirus (murine hepatitis virus strain A59) is followed by functional recovery and efficient CNS myelin repair. To study the biological properties of the cells involved in this repair process, glial cells were isolated and cultured from spinal cords of these young adult mice during demyelination and remyelination. Using three-color immunofluorescence combined with [<sup>3</sup>H]thymidine autoradiography, we have analyzed the antigenic phenotype and mitotic potential of individual glial cells. We identified oligodendrocytes with an antibody to galactocerebroside, astrocytes with an antibody to glial fibrillary acidic protein, and oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells with the O4 antibody. Cultures from demyelinated tissue differed in several ways from those of age-matched controls: first, the total number of O-2A lin-

age cells was strikingly increased; second, the O-2A population consisted of a higher proportion of O4-positive astrocytes and cells of mixed oligodendrocyte-astrocyte phenotype; and third, all the cell types within the O-2A lineage showed enhanced proliferation. This proliferation was not further enhanced by adding PDGF, basic fibroblast growth factor (bFGF), or insulin-like growth factor I (IGF-I) to the defined medium. However, bFGF and IGF-I seemed to influence the fate of O-2A lineage cells in cultures of demyelinated tissue. Basic FGF decreased the percentage of cells expressing galactocerebroside. In contrast, IGF-I increased the relative proportion of oligodendrocytes. Thus, O-2A lineage cells from adult mice display greater phenotypic plasticity and enhanced mitotic potential in response to an episode of demyelination. These properties may be linked to the efficient remyelination achieved in this demyelinating disease.

**I**N demyelinating diseases, damage to myelin sheaths disrupts conduction of electrical impulses along axonal processes of neurons. Typically, damaged myelin is not efficiently repaired in the human CNS (Perier and Gregoire, 1965; Prineas et al., 1984). Thus patients with CNS demyelinating diseases, such as multiple sclerosis, frequently experience prolonged neurological dysfunction. In contrast, efficient remyelination and functional recovery is achieved in rodents in certain experimental models of CNS demyelination (reviewed in Ludwin, 1981). One such model is produced by infecting C57Bl/6N mice with a coronavirus (murine hepatitis virus strain A59; MHV-A59), which leads to the development of multiple CNS demyelinating lesions (Lavi et al., 1984; Jordan et al., 1989). This coronavirus replicates in glial cells during an early phase of the disease and is subsequently cleared from the CNS (Jordan et al., 1989). Infected mice exhibit clinical signs of CNS demyelin-

ation within the first week post infection (wpi).<sup>1</sup> By 3–5 wpi, focal areas of demyelination are present throughout the CNS, with prominent lesions in the spinal cord. In the following weeks, remyelination is paralleled by functional recovery. Using this model, we can analyze the processes involved in regeneration of the cells that form CNS myelin.

Myelination in the developing CNS requires the coordinated interaction of several types of glial cells (reviewed in Raine, 1984; Wood and Bunge, 1984; Raff, 1989). In vitro studies of optic nerve from newborn rat have characterized a bipotential progenitor cell that can give rise to oligodendrocytes or to type 2 astrocytes, and is therefore called an oligodendrocyte-type 2 astrocyte (O-2A) progenitor cell. Type 2 astrocytes express antigens that are also present on the surface of O-2A progenitor cells. The localization and function of type 2 astrocytes in vivo is not clear at the present time. Another type of astrocyte, called the type 1 astrocyte, appears before type 2 astrocytes and arises from a glial

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1. *Abbreviations used in this paper:* div, days in vitro; GC, galactocerebroside; GFAP, glial fibrillary acidic protein; MHV-A59, murine hepatitis virus strain A59; O-2A, oligodendrocyte-type 2 astrocyte; wpi, weeks post infection.

precursor distinct from the O-2A progenitor (Raff et al., 1984). Type 1 astrocytes secrete growth factors that affect the proliferation, migration, and/or differentiation of O-2A progenitors (reviewed in Raff, 1989; Dubois-Dalcq and Armstrong, 1990). Upon maturation, oligodendrocytes elaborate extensive processes which wrap around CNS axons to form myelin (reviewed in Raine, 1984). Perinodal astrocytes, possibly type 2 astrocytes, extend processes to nodes of Ranvier, which are electrically excitable regions of axons between adjacent myelin sheaths (French-Constant and Raff, 1986b; French-Constant et al., 1986). Both oligodendrocytes and perinodal astrocytes seem to play a role in efficient saltatory conduction of nerve impulses; myelin acts as an electrical insulator while perinodal astrocytic processes may serve various functions associated with impulse propagation at nodes (discussed in Ritchie, 1984; Black and Waxman, 1988).

In normal adult CNS oligodendrocytes proliferate minimally (McCarthy and Leblond, 1988). Attempts to induce *in vitro* mitosis of oligodendrocytes with exogenous growth factors have not been successful (Yong et al., 1988). However, oligodendrocytes isolated from adult CNS do proliferate when cocultured with neurons (Wood and Bunge, 1986). In addition, O-2A progenitor cells isolated from adult rat optic nerve are capable of proliferating *in vitro* in the absence of neurons and maintain the ability to differentiate (French-Constant and Raff, 1986a; Wolswijk and Noble, 1989). These studies provide evidence that oligodendrocytes and/or O-2A progenitor cells may contribute to the regeneration of O-2A lineage cells in the adult CNS.

After widespread CNS demyelination, surviving oligodendrocytes at the periphery of lesions do not seem to be able to elaborate myelinating processes and/or relocate to achieve efficient remyelination. Proliferation of oligodendrocytes, or oligodendrocyte precursor cells, during demyelination appears to be necessary to facilitate remyelination. Electron microscopic autoradiographic analyses of demyelinating tissue have reported mitosis of oligodendrocytes at several stages of maturation (Herndon et al., 1977; Ludwin, 1979; Aranella and Herndon, 1984; Ludwin and Bakker, 1988). A recent *in vivo* analysis combining immunolabeling with autoradiography has shown that O-2A progenitor cells and astrocytes that express O4 antigens proliferate early in the course of demyelination induced by coronavirus infection and that some cells generated during demyelination later became oligodendrocytes (Godfraind et al., 1989). In the present study, we have developed an *in vitro* system to further characterize the cells involved in the remyelination process. We have used three-color immunofluorescence combined with autoradiography to identify specific glial cell types. In such a system we can analyze the proliferative capacity, phenotypic plasticity, and differentiation potential of the various types of glial cells which may play a key role in CNS remyelination.

## Materials and Methods

### Coronavirus Infection

Female C57Bl/6N mice, without prior exposure to MHV-A59, were obtained from the Frederick Cancer Research Facility (Frederick, MD). At 28 d of age each mouse was injected intracranially with 1,000 plaque-forming units of coronavirus (MHV-A59), which had been propagated in the 17 Cl 1 line of spontaneously transformed BALB/c 3T3 cells (Sturman et al., 1980). This age and inoculation dosage optimized the proportion of

mice developing demyelinating lesions (~69%) relative to the incidence of acute mortality (~11%).

### Glial Cell Isolation

At 1, 3, 4, 5, or 8 wpi, mice were anesthetized with methoxyflurane and then decapitated. For each glial cell isolation, spinal cords removed from three infected mice exhibiting neurological dysfunction (Woyciechowska et al., 1984) were combined and spinal cord tissue from three age-matched control mice was prepared simultaneously. Spinal cords were minced and then dissociated according to a procedure modified from Miller et al. (1985). The tissue was incubated at 37°C with enzymes (twice for 20 min in 0.125% trypsin with 0.02% collagenase in MEM-Hepes followed by one 15-min incubation in 0.05% trypsin with 0.53 M EDTA), bathed in a solution that inhibited trypsin and digested free DNA (0.25% soybean trypsin inhibitor, 0.002% DNase I, 0.166% BSA, and 5% FBS in DME) and passed through pipettes (5 ml, 10 times; 1 ml, 10 times; Pasteur pipette 10 times). Floating cells were transferred to a 50-ml tube which was then filled with isolation medium (1 mM Hepes, 50 U/ml penicillin, 50 µg/ml streptomycin, and 25 µg/ml gentamycin in HBSS without calcium or magnesium, pH 7.4) and spun for 10 min at 1,500 rpm in a centrifuge (GLC-2B; Sorvall Div., Newton, CT). The supernatant was removed and pelleted cells were resuspended in 10 ml of isolation medium.

Glial cells were separated from myelin and red blood cells using a 30% Percoll gradient (Hirayama et al., 1983; Kim et al., 1985). The gradient was centrifuged in an ultracentrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JA20 fixed-angle rotor spun at 14,000 rpm for 45 min at 4°C. The glial cell layer, the region below the myelin cap and above the red blood cells, was transferred to a 50-ml tube that was then filled with isolation medium and spun for 10 minutes at 1,500 rpm in a centrifuge (GLC-2B; Sorvall Div.). The supernatant was removed and the pelleted cells were resuspended (800 µl/spinal cord) in 10% FBS in DME with 584 mg/liter L-glutamine, 4.5 g/liter D-glucose, 25 µg/ml gentamycin, and 1 mM sodium pyruvate. In all cases, 200 µl of cell suspension was plated per 35-mm plastic dish (coated with extracellular matrix; Accurate Chemical & Scientific Corp., Westbury, NY). After incubating for 1 h at 37°C to enhance attachment, the cultures were fed with 2 ml of 10% FBS-DME solution. At 1 and 3 d *in vitro* (div) cultures were fed with 0.5% FBS in defined medium (DME with 584 mg/liter L-glutamine, 4.5 g/liter D-glucose, 5 µg/ml insulin, 50 µg/ml transferrin, 30 nM selenium, 30 nM triiodothyronine, 25 µg/ml gentamycin, and 1 mM sodium pyruvate; modified from Eccleston and Silberberg, 1984). The cultures were grown in this low serum defined medium to enhance the growth of oligodendrocytes relative to fibroblasts. After 1, 3, or 6 div, cultures were fixed with 2% paraformaldehyde in MEM-Hepes for 15 min.

### [<sup>3</sup>H]Thymidine Labeling

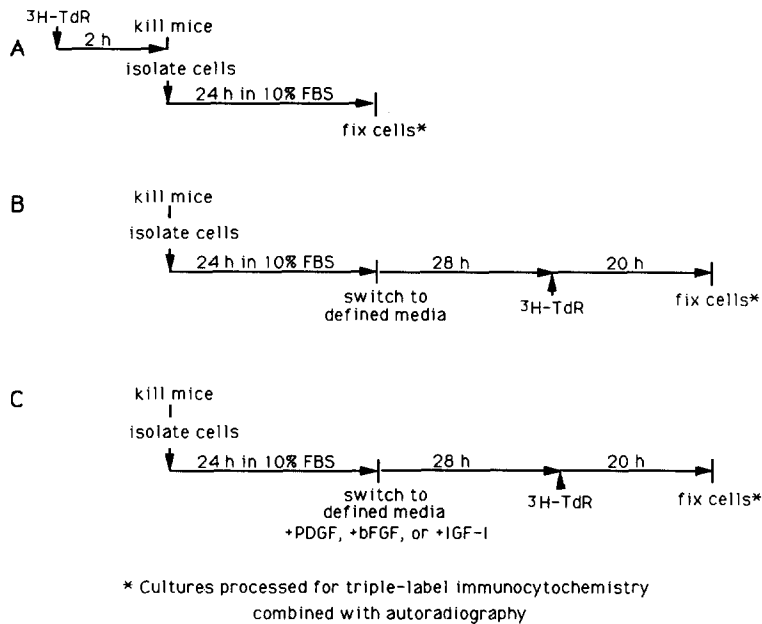
In some experiments a terminal pulse of [*methyl*-<sup>3</sup>H]thymidine (67 Ci/mmol; New England Nuclear, Boston, MA) was administered. For *in vivo* labeling, mice were injected intraperitoneally with [<sup>3</sup>H]thymidine (10 µCi/g body weight) 2 h before death (see Fig. 1 A). For *in vitro* labeling, [<sup>3</sup>H]thymidine (0.05 µCi/ml culture media) was added to cultures 20 h before fixation (see Fig. 1 B).

### Growth Factors

The growth factors tested were bovine bFGF (10 ng/ml; R & D Systems, Minneapolis, MN), human PDGF (10 ng/ml; A and B chain heterodimer from R & D Systems), and human insulin-like growth factor I (IGF-I; 100 ng/ml; Amgen Biologicals, Thousand Oaks, CA). Each growth factor was added individually to a culture dish at 1 div with 0.5% FBS in defined medium (see above). Cultures were subsequently given a 20-h terminal pulse of [<sup>3</sup>H]thymidine and fixed at 3 div (see Fig. 1 C).

### Immunocytochemistry

O-2A lineage cells were identified based upon three-color immunofluorescence that enabled simultaneous visualization of reactivity with anti-galactocerebroside (GC), anti-glial fibrillary acidic protein (GFAP), and the O4 antibody. Anti-GC is a mouse monoclonal IgG<sub>3</sub>, kindly provided by B. Ranscht (La Jolla Cancer Research Foundation, La Jolla, CA), which recognizes GC and an earlier antigen emerging on the cell surface shortly after the appearance of O4 immunostaining (Ranscht et al., 1982; Bansal et al., 1989). The rabbit polyclonal anti-GFAP, kindly provided by R. Pruss (Merrill Dow Pharmaceuticals, Cincinnati, OH), immunostains GFAP but



**Figure 1.** Experimental design followed for the in vivo and in vitro [ $^3\text{H}$ ]thymidine labeling studies of O-2A lineage cells and their response to growth factors.

does not react with other intermediate filament proteins (Pruss et al., 1979). O4 is a mouse monoclonal IgM, kindly provided by I. Sommer (Southern General Hospital, Glasgow, Scotland), (Sommer and Schachner, 1981) which recognizes sulfatide, seminolipid, and an unidentified antigen (Bansal et al., 1989).

O4 and anti-GC were supernatants from hybridoma cultures containing 10% FBS in DME. The supernatants were mixed together and diluted 1:4 and 1:2, respectively, in a MEM-Hepes buffer solution (0.1% gelatin, 1% BSA, and 0.05% sodium azide in MEM-Hepes) and applied to the fixed cells for 1–2 h. O4 was visualized with rhodamine conjugated goat anti-mouse IgM (25  $\mu\text{g}/\text{ml}$ ; Jackson Immunoresearch Laboratories, West Grove, PA). Anti-GC was visualized with fluorescein conjugated goat anti-mouse IgG<sub>3</sub> (16.6  $\mu\text{g}/\text{ml}$ ; Fischer Biotech, Orangeburg, NY). These secondary antibodies were also mixed together in MEM-Hepes buffer solution and applied for 1–2 h. After washing, the cultures were fixed with 5% glacial acetic acid in ethanol for 10 min at  $-20^\circ\text{C}$  to expose internal cellular antigens. This was followed by three washes in 10% FBS MEM-Hepes and three washes in 0.5M Tris buffer. Polyclonal rabbit anti-GFAP was diluted 1:2,000 in a Tris buffer solution (0.1% gelatin, 1% BSA, and 0.05% sodium azide in 0.5M Tris buffer) for binding overnight at  $4^\circ\text{C}$ . Anti-GFAP was visualized with biotinylated donkey anti-rabbit IgG (10  $\mu\text{g}/\text{ml}$  in the Tris buffer solution, 1–2 h; Amersham Chemical Co., Arlington Heights, IL) followed by streptavidin conjugated 7-amino-4-methyl-coumarin-3-acetic acid (25  $\mu\text{g}/\text{ml}$  in the Tris buffer solution, 1–2 h; Molecular Probes, Inc., Eugene, OR; Khalifan et al., 1986). After a final 5-min fixation in 4% paraformaldehyde the cultures were either coverslipped, with a solution of 20% 0.02 M Tris buffer (pH 8.2) and 80% glycerol, or processed for autoradiography (see below).

Immunostained cells were examined by epifluorescence with a Zeiss Photoscope III equipped with three filter sets to simultaneously view rhodamine, fluorescein, and coumarin fluorescence. The absence of cross-reaction in the three-color immunofluorescence protocol was tested by omitting, in turn, each one of the three primary antibodies while all other steps remained unchanged. Omission of each primary completely abolished binding of the corresponding secondary but did not affect signals in the other two channels.

In some cultures, the presence of mixed oligodendrocyte-astrocyte phenotype cells was confirmed with a second set of primary antibodies. These cultures were immunostained with O4, as described above, in combination with a rat mAb to GFAP (Lee et al., 1984) visualized with fluorescein and a rabbit polyclonal antiserum to GC, which was produced in our laboratory (according to the procedure described by Benjamins et al., 1987), and visualized with coumarin.

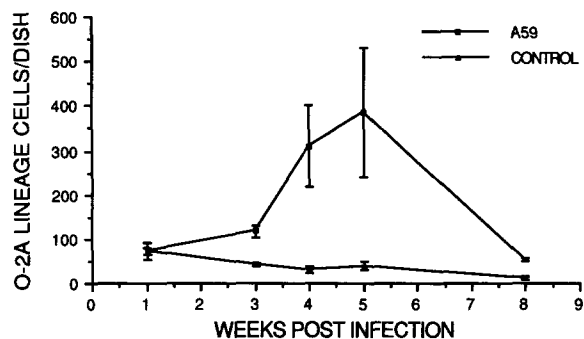
### Histological Sections

A segment of each spinal cord was excised with a sterile razor blade before glial cell isolation. Segments were fixed by overnight immersion in 1%

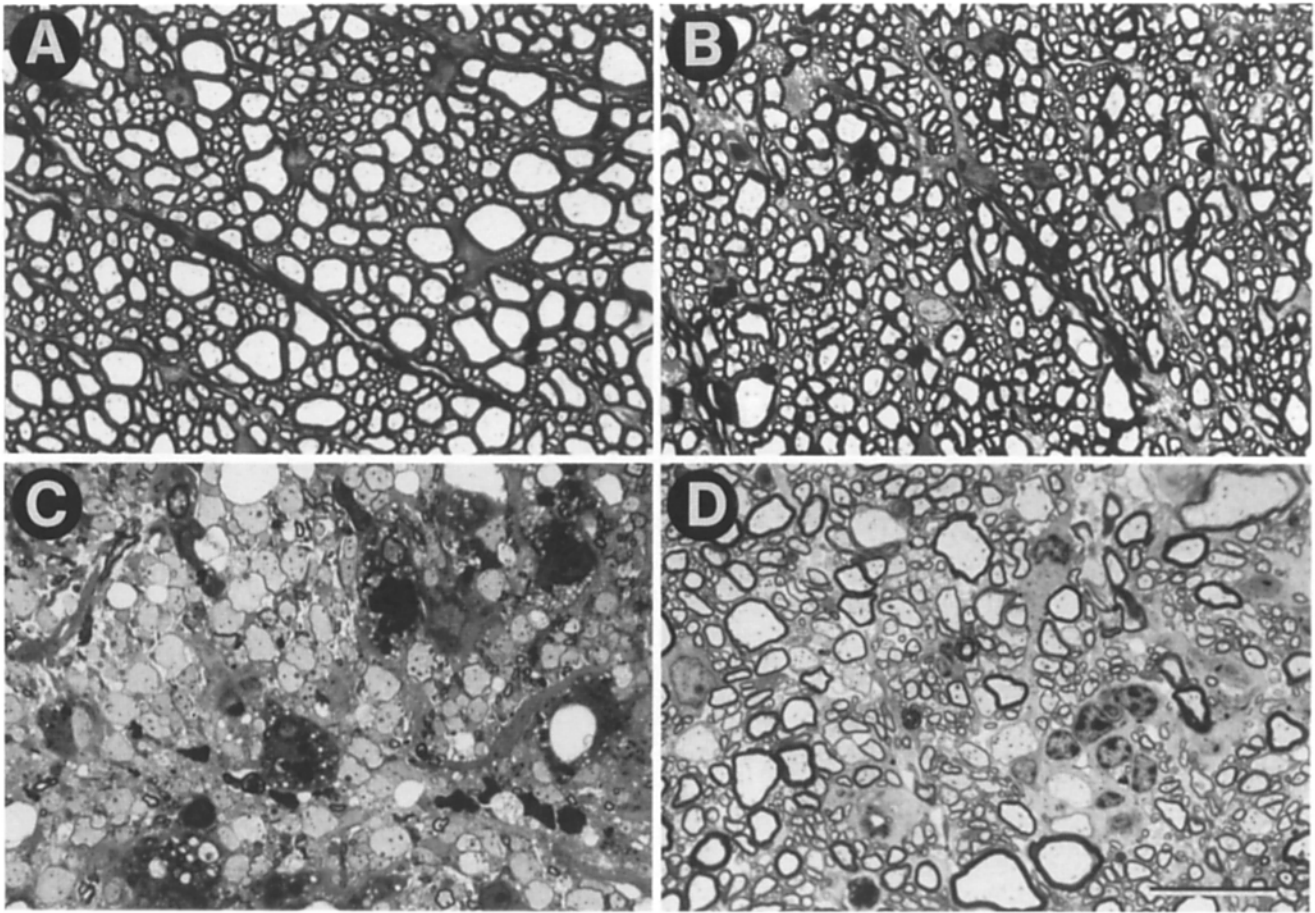
glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. The tissue was then washed for 1 d in buffer, postfixed with 2% osmium tetroxide for 2 h, dehydrated in graded alcohols, cleared with propylene oxide, and embedded in Epon resin. Transverse sections (1  $\mu\text{m}$ ) were stained with toluidine blue.

### Autoradiography

Cultured cells, labeled either in vivo or in vitro with [ $^3\text{H}$ ]thymidine, were immunostained and post-fixed as described above. The specimens were then dehydrated in graded alcohols (50%, 75%, 95%; 30 seconds in each), air dried, coated with Kodak NTB2 nuclear track emulsion (diluted 1:2), exposed at  $4^\circ\text{C}$  for 5 d, developed at  $16^\circ\text{C}$  in Kodak D19, and fixed with Kodak fixer. After several washes, cultures were coverslipped as described above.



**Figure 2.** Glial cells isolated from spinal cords of diseased (■) and control (▲) mice were cultured for 1 d and then O-2A lineage cells were identified by triple-label immunocytochemistry (as described in Results). The total number of O-2A lineage cells (oligodendrocytes, O-2A progenitors, type 2 astrocytes, and mixed phenotype cells) in each 35-mm dish was counted. The number of O-2A lineage cells cultured from demyelinated tissue (3–5 wpi, see Fig. 3 C) is significantly greater than the number from control tissue (3 wpi,  $p = 0.015$ ; 4 wpi,  $p = 0.019$ ; 5 wpi,  $p = 0.069$ ; paired two-tailed  $t$  test). For each glial cell isolation, the pellet from 3 spinal cords was plated onto 12 dishes. Each value represents the average of three to seven dishes combined from up to four series of animals. Error bars = SEM. In all, 5,988 O-2A lineage cells were counted.



**Figure 3.** A small segment of fresh tissue was excised from each spinal cord and processed for histological examination while the rest of the spinal cord was dissociated for *in vitro* studies. The progression of demyelination and remyelination was followed in 1  $\mu\text{m}$  epoxy-embedded sections stained with toluidine blue. (A) In control tissue, intact myelinated axons are tightly packed within the white matter. (B) At 1 wpi, demyelination is not yet detected, although occasionally discrete lesions are present (not shown). (C) At 5 wpi, extensive myelin loss is accompanied by vacuolation and phagocytosis. (D) By 8 wpi, abnormally thin myelin sheaths indicate that remyelination is proceeding. Bar, 20  $\mu\text{m}$ .

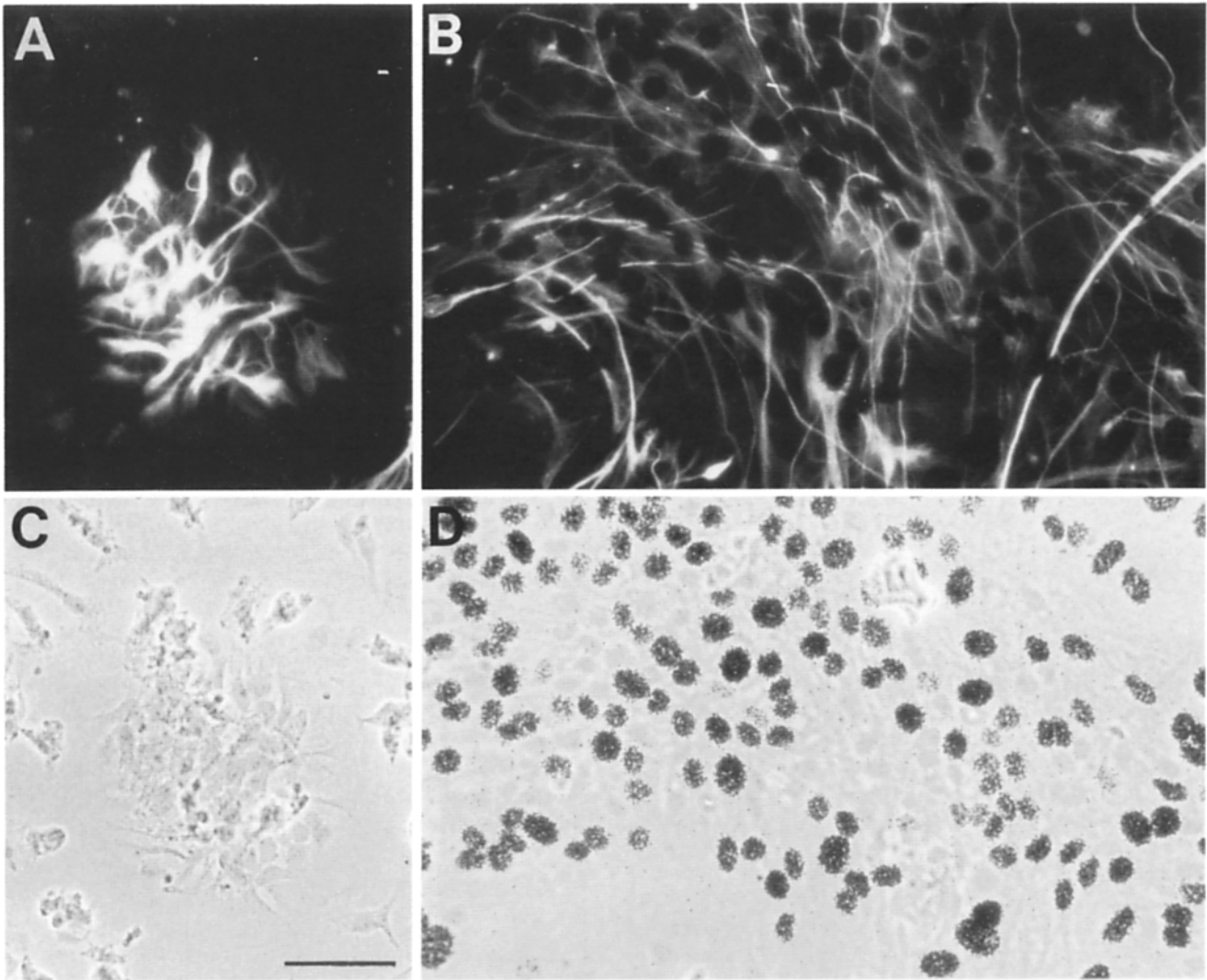
## Results

### *Glial Cell Types Isolated from Control and from Infected Spinal Cord Tissue*

The glial cell population was characterized using three-color immunofluorescence which enabled simultaneous visualization of three antigens, each labeled by one of three different fluorochromes (rhodamine, fluorescein, or coumarin). Oligodendrocytes were identified by expression of cell surface antigens recognized by anti-GC (Raff et al., 1978). Cells containing intracellular filaments immunostained with anti-GFAP were classified as astrocytes (Bignami et al., 1972; Raff et al., 1979). Type 2 astrocytes were distinguished from type 1 astrocytes by the expression of cell surface antigens recognized by the O4 antibody (Trotter and Schachner, 1989). O-2A progenitors derived from adult CNS were identified as cells binding O4 in the absence of GC or GFAP immunolabeling (Wolswijk and Noble, 1989). Thus, any particular cell could be identified as an oligodendrocyte (O4+ GC+ GFAP-), a type 2 astrocyte (O4+ GFAP+ GC-), an O-2A progenitor (O4+ GC- GFAP-), a mixed oligodendrocyte-astrocyte phenotype cell (O4+ GC+ GFAP+), or

a type 1 astrocyte (GFAP+ O4- GC-). (In this paper, the term "type 2 astrocyte" will be used to denote only the O4+ GFAP+ GC- antigenic phenotype and is not intended to indicate a specific localization or function *in vivo*.)

Glial cells were isolated and cultured in parallel from spinal cords of infected and age-matched control mice at several intervals after viral inoculation. These cultures contained various types of glial cells, of which O-2A lineage cells (oligodendrocytes, O-2A progenitors, type 2 astrocytes, and mixed phenotype cells) represented a small proportion of the total. The number of O-2A lineage cells was counted after 1 d in culture (Fig. 2). This number was similar for control and experimental tissue at 1 wpi (Fig. 2) when demyelination was not yet detected (see Fig. 3, A and B). As demyelination and vacuolation progressed (3–5 wpi, see Fig. 3 C), O-2A lineage cells were much more abundant in cultures of the demyelinated tissue (Fig. 2). With the onset of remyelination (6–8 wpi, see Fig. 3 D), the number of O-2A lineage cells derived from lesioned tissue declined (Fig. 2). It is possible that CNS inflammation and vacuolation may have facilitated dissociation of the demyelinated tissue and thereby improved the yield of growing cells. Yet the cultures from control versus demyelinated CNS differed not only in cell



**Figure 4.** At 1 div, type 1 astrocytes stained with anti-GFAP antibodies (*A*) (but not by O4 and GC antibodies, not shown) form the dense clusters of cells seen by phase microscopy (*C*). After 6 d in culture GFAP+ cells have spread out from the clusters (*B*) and proliferated, as assayed by the high proportion of nuclei which have incorporated [<sup>3</sup>H]thymidine during the 3-d terminal pulse (*D*; same field as shown in *B*). [<sup>3</sup>H]Thymidine-labeled GFAP+ cells were also evident at 3 div after a 20-h pulse (not shown). Bar, 50  $\mu$ m.

number but also in antigenic phenotypes and mitotic potential of the O-2A lineage cells.

The other cell types in our cultures were type 1 astrocytes, fibroblasts, and microglia. Flat cells expressing GFAP but not O4 antigens were identified as type 1 astrocytes. Type 1 astrocytes seemed to be more prevalent in cultures of lesioned tissue. However, at 1 div such cells were usually found in dense clusters that prohibited accurate quantitation of the initial type 1 astrocyte population (Fig. 4, *A* and *C*). By 3 and 6 div, type 1 astrocytes grew out from these clusters and proliferated, as assayed by [<sup>3</sup>H]thymidine incorporation (Fig. 4, *B* and *D*). Flat cells presumed to be fibroblasts, due to the absence of O4, GC, or GFAP immunostaining, were present and proliferated in all cultures examined (not shown).

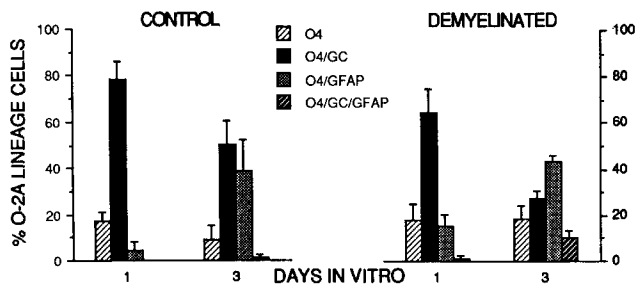
Microglia and/or macrophages were identified by their amoeboid appearance, the presence of phagocytic debris within the cells, and the absence of O4 immunostaining (Giulian, 1987; Rieske et al., 1989). Microglial cells are the resident macrophages of the CNS and markers for distinguishing mi-

croglia from blood-born macrophages, which can infiltrate the CNS, are not presently available. For simplicity, cells with the phenotype of microglia and/or macrophages will be referred to as microglia throughout this paper. These cells were rare in cultures of control tissue. In contrast, the number of microglia cultured from diseased tissue increased dramatically during the period of demyelination (3–5 wpi).

We identified Schwann cells with an antibody to the receptor for nerve growth factor (Chandler et al., 1984) that labeled Schwann cells cultured from rat sciatic nerve, which have been shown to express this receptor (Yasuda et al., 1987; DiStefano and Johnson, 1988). These cells were not detected in cultures of control or infected spinal cords (1 and 4 wpi). Similarly, Schwann cells were rarely observed in the remyelinating lesions (Fig. 3).

#### *In Vitro Phenotypes of O-2A Lineage Cells*

We then examined the relative abundances of the different



**Figure 5.** Relative abundance of specific phenotypes within the O-2A lineage population in cultures derived from demyelinated tissue (5 wpi) and those from age-matched controls. Using triple-label immunocytochemistry, individual cells were identified as O-2A adult progenitors (O4), oligodendrocytes (O4/GC), type 2 astrocytes (O4/GFAP), or mixed phenotype cells (O4/GC/GFAP) based upon which of the three glial cell markers were expressed. Cells of each phenotype were counted and the percentage of each within the total O-2A lineage population was calculated. Cultures of demyelinated tissue contained a higher proportion of type 2 astrocytes and mixed phenotype cells than did control cultures. Each value represents the average of five dishes combined from three separate series of animals. Error bars = SEM. A total of 3,913 O-2A lineage cells were counted.

phenotypes within the O-2A lineage in cultures from control and demyelinated spinal cords (Fig. 5). In control cultures, the O-2A lineage population consisted primarily of oligodendrocytes (GC+ O4+ GFAP-) (Figs. 5 and 6). O-2A progenitor cells (O4+ GC- GFAP-) were found at both 1 and 3 div in these control cultures (Figs. 5 and 7). The relative proportion of type 2 astrocytes (O4+ GFAP+ GC-) increased between 1 and 3 div, even though the cultures were maintained in defined medium with only 0.5% FBS (Figs. 5 and 7). Interestingly, in cultures of neonatal rat optic nerve, 10% FBS is required to induce development of a substantial number of type 2 astrocytes (Raff et al., 1983).

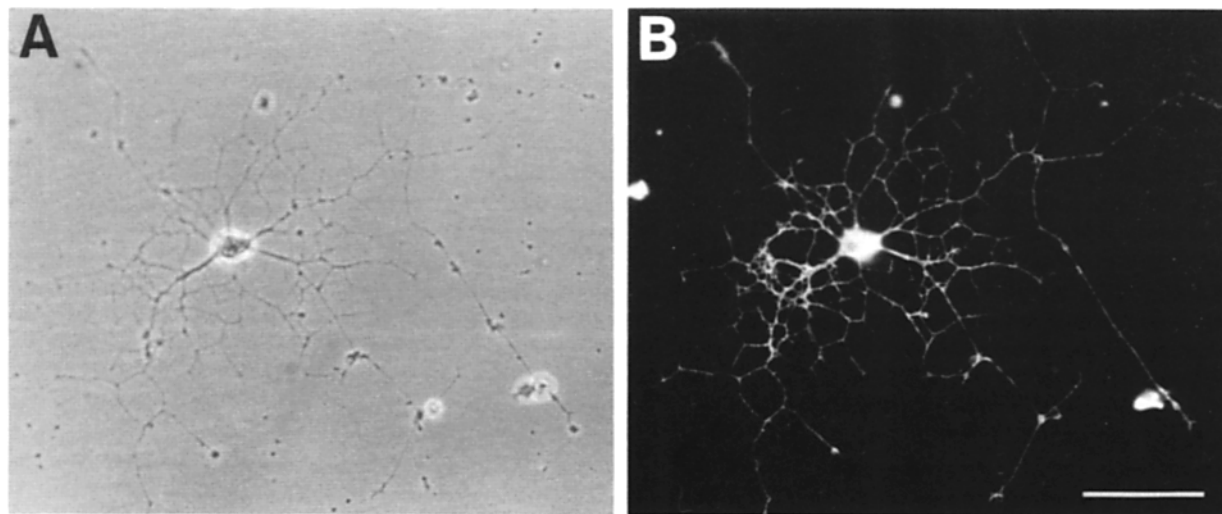
In cultures of demyelinated spinal cord tissue (5 wpi),

oligodendrocytes were the predominant cell type after 1 div (Fig. 5). However, a larger percentage of the O-2A population was composed of type 2 astrocytes in these cultures as compared with cultures of control tissue. By 3 div, type 2 astrocytes became even more prevalent than oligodendrocytes. At each time point, O-2A progenitor cells represented ~20% of the O-2A lineage population. A remarkable feature in cultures from demyelinated tissue was the presence of mixed oligodendrocyte-astrocyte phenotype cells, which were rarely seen in control cultures (Fig. 5). These cells expressed GC on their surface and contained intracellular filaments immunostained with GFAP (Fig. 8). Such mixed phenotype cells (O4+ GC+ GFAP+) were found consistently using two different antibodies to GC in combination with two different antibodies to GFAP (see Materials and Methods).

Certain growth factors have been shown to have an effect on the fate of O-2A lineage cells: IGF-I promotes oligodendrocyte development (McMorris et al., 1986), whereas bFGF inhibits myelin gene expression (McKinnon and Dubois-Dalq, 1990). Therefore, we examined whether treatment with such growth factors from 1-3 div would influence the antigenic phenotypes expressed by O-2A lineage cells isolated during the course of demyelination and remyelination (Fig. 9). In cultures of demyelinating/remyelinating tissue exogenous IGF-I increased the proportion of oligodendrocytes relative to type 2 astrocytes, whereas bFGF decreased the relative number of oligodendrocytes. PDGF did not markedly alter the ratio of type 2 astrocytes to oligodendrocytes, in agreement with the observation that PDGF allows timely differentiation of oligodendrocytes and type 2 astrocytes in cultures of developing CNS tissue (Noble et al., 1988; Raff et al., 1988).

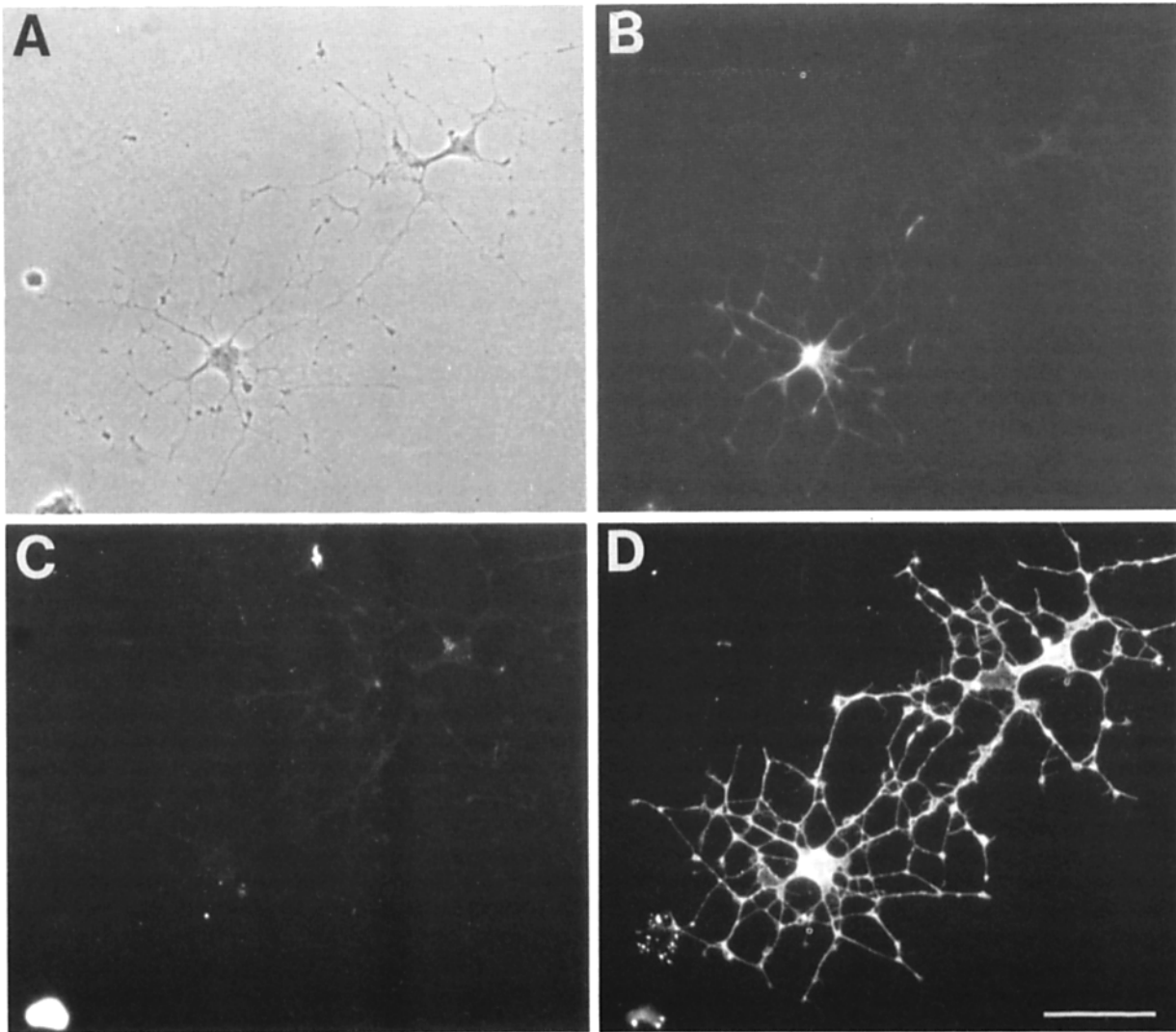
#### *In Vivo and In Vitro Proliferation of O-2A Lineage Cells*

In a previous *in vivo* study of this demyelinating disease, O-2A lineage cells which had incorporated [<sup>3</sup>H]thymidine during a 2-h terminal pulse were detected in 1- $\mu$ m frozen sec-



**Figure 6.** An example of an oligodendrocyte isolated from normal adult (8-wk-old) mouse spinal cord which was grown in culture for 3 d, fixed, and processed for three-color immunofluorescence. (A) Phase contrast showing the thin, branched processes which are characteristic of an oligodendrocyte. (B) This cell is labeled with galactocerebroside antibody, which is visualized with fluorescein. This cell also bound O4 but did not express GFAP (not shown). Bar, 50  $\mu$ m.

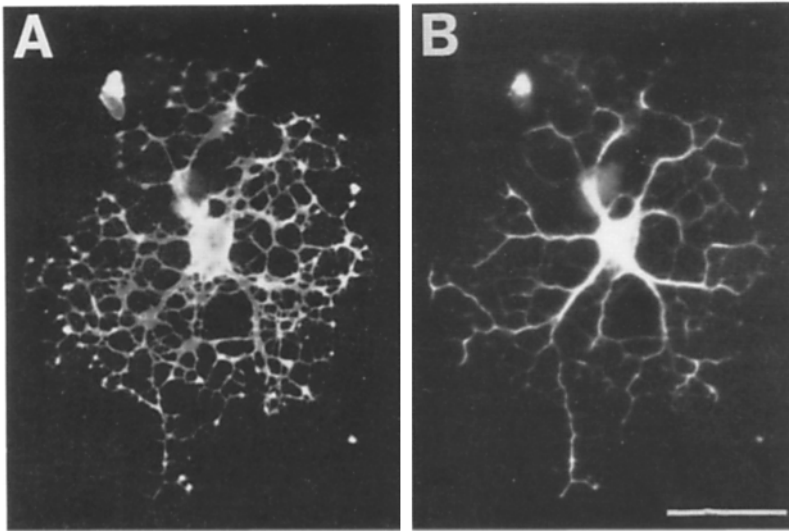




**Figure 7.** A type 2 astrocyte (O4+ GFAP+ GC-) and an O-2A progenitor (O4+ GFAP- GC-) from normal adult (8-wk-old) mouse spinal cord which were grown in culture for 3 d, fixed, and processed for three-color immunofluorescence. Phase contrast reveals two process-bearing cells (A). The type 2 astrocyte in the bottom left quadrant (A) has intracellular filaments stained with GFAP antiserum (B; coumarin optics) and also expresses surface antigens recognized by the O4 antibody (D; rhodamine optics) but not the GC antibody (C; fluorescein optics). The adult progenitor cell in the upper right quadrant (A) expresses O4 antigens (D) but not GFAP (B) or GC (C). Bar, 50  $\mu$ m.

tions (Godfraind et al., 1989). To further explore the mitotic potential of O-2A lineage cells from adult CNS, we prepared cultures from spinal cords of normal and infected mice after *in vivo* labeling with [ $^3$ H]thymidine during a 2-h terminal pulse (Table I). Cells undergoing active DNA synthesis were identified after 1 div by [ $^3$ H]thymidine autoradiography combined with triple-label immunocytochemistry, as outlined in Fig. 1 A. The proportion of O-2A lineage cells (oligodendrocytes, O-2A progenitors, and type 2 astrocytes) which were thymidine-labeled was extremely low in control cultures prepared from normal mice at 5 wk of age (0.52%) and at 8 wk of age (0%). In contrast, the proportion of O-2A lineage cells (oligodendrocytes, O-2A progenitors, type 2 astrocytes, and mixed phenotype cells) that were [ $^3$ H]thymidine-labeled in cultures derived from infected tissue was markedly higher both in the early phase of the disease (4.96% at 1 wpi; 5 wk of age) and at the time of widespread demyelination (5.38% at 4 wpi; 8 wk of age).

We next examined the proliferative capacity of the cultured cells during an *in vitro* pulse of [ $^3$ H]thymidine (Table I). In this case proliferation *in vitro* was measured by adding [ $^3$ H]thymidine to the culture medium at 2 div, fixing the cells 20 h later, and then identifying [ $^3$ H]thymidine-labeled cells by autoradiography combined with three-fluorochrome immunocytochemistry, as outlined in Fig. 1 B. The percentage of O-2A lineage cells that were labeled *in vitro* with [ $^3$ H]thymidine was approximately ninefold higher in cultures derived from diseased mice at 1 wpi than in cultures from age-matched control mice, and was increased more than threefold at 4 wpi (Table I). Oligodendrocytes (Fig. 10), type 2 astrocytes (Fig. 11), O-2A progenitors (Fig. 12), and mixed phenotype cells were labeled with [ $^3$ H]thymidine after the 20-h terminal pulse. Remarkably, O-2A progenitors were the only cell type for which the increased percentage of [ $^3$ H]thymidine-labeled cells from demyelinated tissue was highly significant (4 wpi; demyelinated = 38.3%, control =



**Figure 8.** A cell with a mixed oligodendrocyte-astrocyte phenotype isolated from demyelinated tissue (4 wpi) which was grown in culture for 3 d, fixed, and processed for three-color immunofluorescence. (A) The cell surface membrane is immunostained with an mAb against GC, visualized with fluorescein. (B) Intracellular filaments are immunostained with a polyclonal antiserum recognizing GFAP, visualized with coumarin. This cell was also labeled by O4, visualized with rhodamine (not shown). Bar, 50  $\mu$ m.

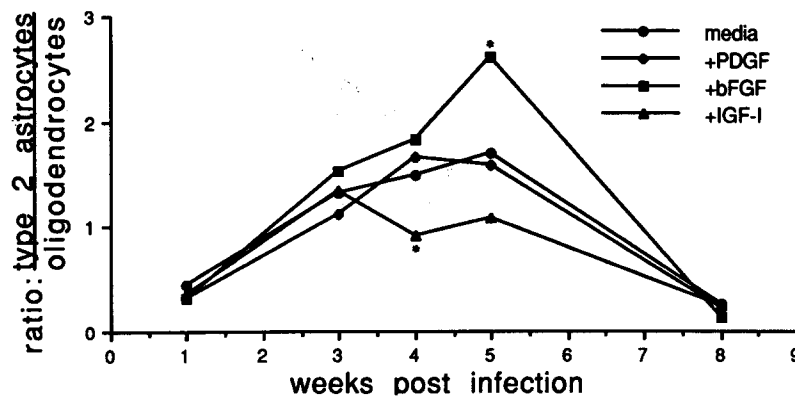
0%;  $p = 0.027$ , as determined by paired two-tailed  $t$  test). Approximately 12–14% of each of the other O-2A lineage cell types (oligodendrocytes, type 2 astrocytes, and mixed phenotype cells) were [ $^3$ H]thymidine-labeled in cultures derived from demyelinated tissue. Although the percentage of [ $^3$ H]thymidine-labeled cells within each of these phenotypes alone was not increased significantly, when combined as a single group these three phenotypes accounted for a significant proliferative response in cultures of demyelinated tissue (4 wpi; demyelinated = 13.5%, control = 4.3%;  $p = 0.029$ , as determined by paired two-tailed  $t$  test). Thus the *in vivo* proliferative response of O-2A lineage cells isolated from demyelinating tissues was retained *in vitro*.

Since PDGF, IGF-I, and bFGF can each induce proliferation of neonatal O-2A progenitor cells in the developing rat CNS (reviewed in Dubois-Dalq and Armstrong, 1990), we assayed the mitogenic effect of these growth factors in our cultures of adult mouse spinal cord (see protocol outlined in Fig. 1 C). We tested each growth factor at 1, 3, 4, 5, and

8 wpi since the effect of a particular growth factor may vary during the course of the disease. In cultures of demyelinating and remyelinating tissue, none of these three growth factors caused a significant increase in the percentage of O-2A lineage cells which incorporate [ $^3$ H]thymidine during a 20-h terminal pulse ( $p = 0.691$  for the difference between treatments, as determined by ANOVA). Similarly, preliminary data from control cultures indicated that treatment with PDGF, IGF-I, or bFGF did not enhance the proliferation of O-2A lineage cells from normal adult mice. Thus, exogenous growth factors influenced phenotype expression (Fig. 9) but did not enhance the proliferation of O-2A lineage cells in our cultures.

## Discussion

In the present *in vitro* study, we have characterized the growth and differentiation of glial cells isolated from the CNS of mice during the course of demyelination and remy-



**Figure 9.** The relative abundance of type 2 astrocytes and oligodendrocytes in spinal cord cultures from virus-inoculated animals. The number of type 2 astrocytes and oligodendrocytes was determined as described in Fig. 5. In defined media alone, the proportion of type 2 astrocytes increases during demyelination (3–5 wpi). Addition of bFGF (10 ng/ml) to the defined media between 1 and 3 div exaggerates this shift toward the type 2 astrocyte phenotype by decreasing the relative number of cells expressing GC. Treatment with IGF-I (100 ng/ml) induces a larger proportion of cells to express the oligodendrocyte phenotype. Exogenous PDGF (10 ng/ml) did not alter the ratio of type 2 astrocytes to oligodendrocytes. To minimize vari-

ability between experiments, the cultures were prepared in parallel for each timepoint and as one completely matched set from mice inoculated at the same time with the demyelinating virus. Each value represents the ratio from cell counts in an entire 35-mm dish. A total of 1,793 cells were counted in the set of cultures. To estimate the variability that might be expected between nonmatched experiments, the ratio of type 2 astrocytes to oligodendrocytes was compared in three similar experiments of cultures grown without exogenous growth factors. The asterisks denote values which fall outside of a 95% confidence interval of the expected variability between experiments for cultures grown without exogenous growth factors.



**Table 1. Percentage of O-2A Lineage Cells Labeled with [<sup>3</sup>H]Thymidine**

Protocol	1 wpi		4 wpi	
	Control	MHV-A59	Control	MHV-A59
2-h in vivo pulse (cells fixed at 1 div)	0.52 ± 0.52	4.96 ± 2.18*	0 ± 0	5.38 ± 2.51‡
20-h in vitro pulse (cells fixed at 3 div)	4.85 ± 1.00	43.81 ± 9.83§	4.68 ± 2.7	17.48 ± 2.48¶

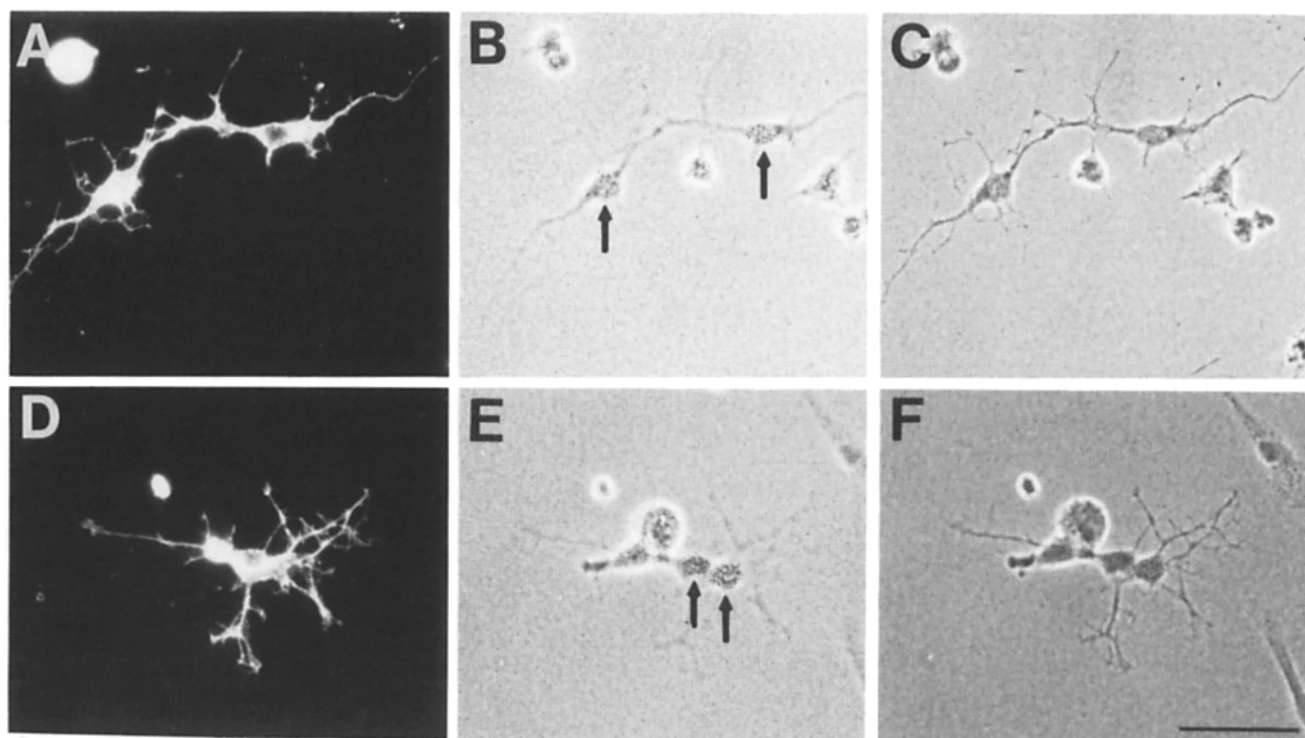
\*  $p = 0.228$ ; ‡  $p = 0.278$ ; §  $p = 0.048$ ; ¶  $p = 0.011$ .

The percentage of dividing O-2A lineage cells in cultures from control and demyelinating tissue (1 and 4 wpi) was determined following either a 2-h in vivo or 20-h in vitro pulse of <sup>3</sup>H-thymidine (see protocols outlined in Fig. 1, A and B). Each value is the percentage (±SEM) of total O-2A lineage cells (oligodendrocytes, type 2 astrocytes, O-2A progenitors, and mixed phenotype cells combined) which were [<sup>3</sup>H]thymidine-labeled (greater than 10 autoradiographic silver grains localized over each nucleus) and represents the average of 2-3 dishes. Significance values were determined using the two-tailed paired *t* test. A total of 2,221 O-2A lineage cells were counted in this set of 6 experiments of control mice (660 cells) matched with 6 experiments of MHV-A59 infected mice (1,561 cells).

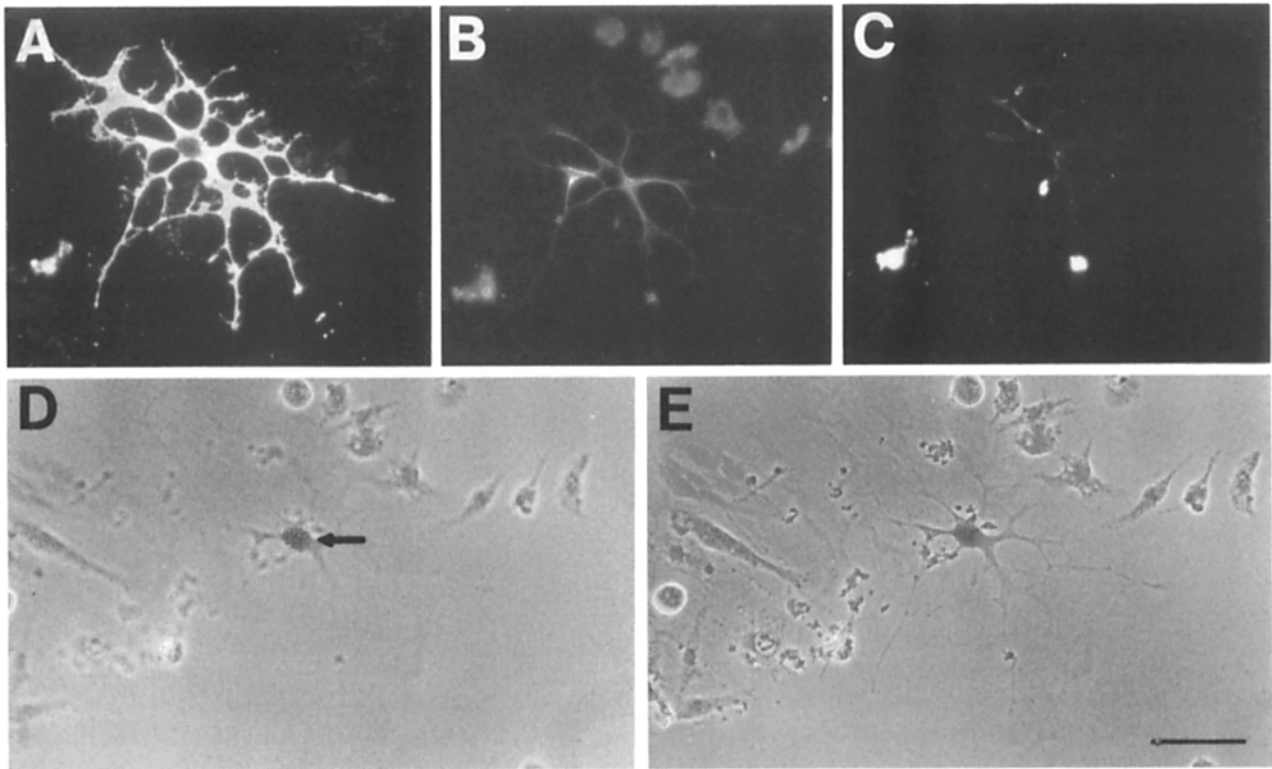
elination. Several differences were observed between the glial cell populations isolated from control and demyelinated spinal cords. First, the number of O-2A lineage cells (oligodendrocytes, O-2A progenitors, type 2 astrocytes, and mixed phenotype cells combined) increased dramatically as demyelination progressed and returned to near control levels as remyelination proceeded. Moreover, O-2A lineage cells derived from demyelinated tissue differed from those of control tissue by the presence of mixed oligodendrocyte-astrocyte phenotype cells and by their differentiation potential in response to exogenous growth factors. Finally, both in vivo and in vitro [<sup>3</sup>H]thymidine labeling demonstrated enhanced proliferation of O-2A lineage cells derived from demyeli-

nated tissue, which correlates well with the increased number of O-2A lineage cells observed in culture. The phenotypic plasticity and mitotic potential O-2A lineage cells cultured from demyelinating/remyelinating tissue may reflect molecular and cellular events critical for efficient remyelination in vivo.

Studies of neonatal CNS tissue have shown that the growth and differentiation of O-2A lineage cells can be influenced in vitro by several polypeptide growth factors, which are synthesized in the brain (for review, see Raff, 1989; Dubois-Dalcq and Armstrong, 1990). PDGF stimulates the proliferation of O-2A progenitor cells, which prevents premature differentiation in vitro (Noble et al., 1988; Raff et al., 1988).



**Figure 10.** Thymidine-labeled oligodendrocytes are found in cultures from demyelinated tissue. These cells were isolated from demyelinated tissue (4 wpi), fixed at 3 div after a 20-h in vitro pulse of [<sup>3</sup>H]thymidine, and processed for autoradiography combined with three-color immunofluorescence. (A and D) The cell surface membranes are immunostained with antibody to GC, visualized with fluorescein (not shown: these cells also expressed O4 but not GFAP). (B and E) Phase contrast focusing in the plane of the autoradiographic emulsion. Arrows point to clusters of silver grains over each nucleus which indicate that these cells incorporated [<sup>3</sup>H]thymidine. (C and F) Phase contrast focusing in the plane of the cell processes. Bar, 50  $\mu$ m.



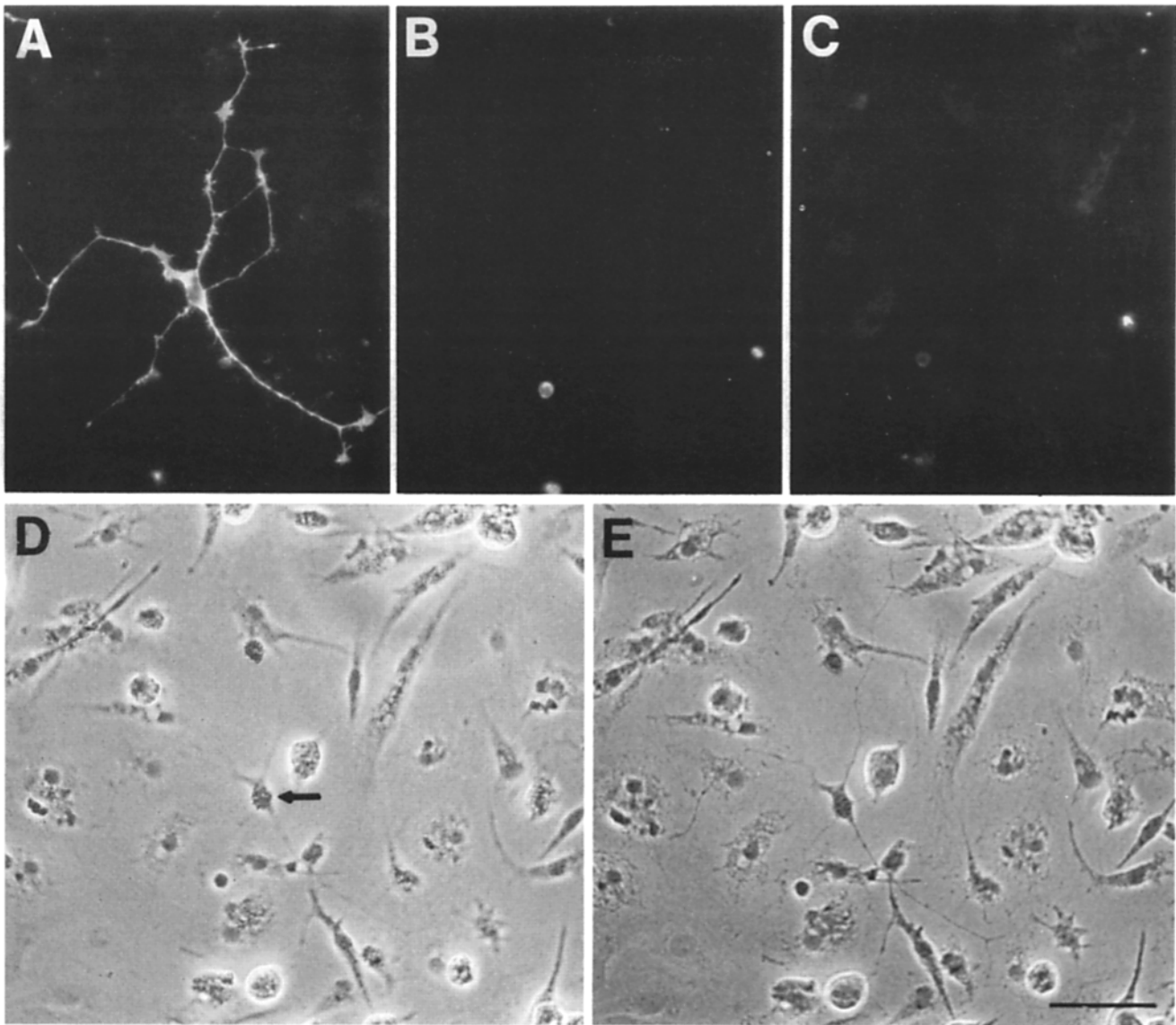
**Figure 11.** A type 2 astrocyte cultured from demyelinated tissue (4 wpi) and processed as described in Fig. 10 is identified by the presence of surface staining with O4 (A; rhodamine optics), intracellular expression of GFAP (B; coumarin optics) and the absence of labeling with GC antibody (C; fluorescein optics). The cluster of silver grains over the nucleus (D, arrow) indicates that this cell incorporated [<sup>3</sup>H]thymidine during the 20-h in vitro pulse. (E) Phase contrast showing the cell processes. Bar, 50  $\mu$ m.

FGF is also mitogenic for O-2A lineage cells (Eccleston and Silberberg, 1985; Noble et al., 1988; Besnard et al., 1989). Both FGF and epidermal growth factor inhibit expression of myelin components (Sheng et al., 1989). IGF-I promotes proliferation of O-2A lineage cells while also inducing precursor cells to develop into oligodendrocytes (McMorris and Dubois-Dalq, 1988). A protein closely related to ciliary neurotrophic factor is present in developing optic nerve and induces transient expression of GFAP in O-2A progenitor cells in vitro (Hughes et al., 1988; Lillien et al., 1988).

Whether the growth factors mentioned above have an effect on O-2A lineage cells derived from adult CNS tissue is not yet clear. Human oligodendrocytes isolated from normal adult brain do not proliferate in response to treatment with several growth factors, including PDGF, FGF, epidermal growth factor, and interleukin 2 (Yong et al., 1988). Similarly, in the present study we found that PDGF, bFGF, or IGF-I did not induce mitosis of O-2A lineage cells cultured from adult mice, as assayed by [<sup>3</sup>H]thymidine incorporation. However, a factor secreted by B104 neuroblastoma cells is mitogenic for O-2A progenitors and oligodendrocytes cultured from adult rat brain (Hunter et al., 1988). Thus, normal adult O-2A lineage cells are capable of proliferating when exposed to an adequate stimulus. Such a stimulus could be a factor (or factors) released in the CNS during demyelination since O-2A lineage cells in the spinal cord of MHV-A59 infected mice showed increased proliferation both in vivo (Godfraind et al., 1989) and in vitro (the present data). Interestingly, some growth factors can influence the

antigenic phenotypes of O-2A lineage cells in our cultures of demyelinating/remyelinating CNS. Basic FGF decreased the percentage of O-2A lineage cells expressing GC, whereas IGF-I increased the proportion of oligodendrocytes relative to type 2 astrocytes. Thus bFGF and IGF-I might modulate phenotype expression in CNS development and remyelination.

Proliferation of mature and immature oligodendrocytes in vivo has been described in several electron microscopic and autoradiographic studies of experimental demyelination in adult CNS (Herndon et al., 1977; Ludwin, 1979; Aranella and Herndon, 1984; Raine et al., 1988). In our in vivo studies of remyelination following coronavirus-induced demyelination in mice, we have used in situ hybridization and immunolabeling techniques to analyze the expression of myelin genes and the presence of cell-type-specific antigens in oligodendrocytes and their precursors (Jordan et al., 1989; Godfraind et al., 1989). We found that myelin basic protein mRNA isoforms containing exon-2, which are normally abundant only during development, are reexpressed at dramatically increased levels during remyelination (Jordan et al., 1990). This recapitulation of molecular events characteristic of development suggests that newly generated oligodendrocytes are responsible for remyelination in this disease. Studies using triple-label immunocytochemistry combined with autoradiography have identified O-2A progenitor cells in 1  $\mu$ m frozen sections of the spinal cord of these infected mice (Godfraind et al., 1989). O-2A progenitors and O4-positive astrocytes proliferated early in the course of the disease and some



**Figure 12.** An O-2A adult progenitor cultured from demyelinated tissue (4 wpi) and processed as described in Fig. 10 is recognized by its reactivity with the O4 antibody (*A*; rhodamine optics) and in the absence of staining with GC (*B*; fluorescein optics) or GFAP (*C*; coumarin optics) antibodies. The cluster of silver grains over the nucleus (*D*, arrow) indicates that this cell incorporated [<sup>3</sup>H]thymidine during the 20-h in vitro pulse. (*E*) Phase contrast showing the cell processes. Note that numerous microglial cells are also present in this culture. Bar, 50  $\mu$ m.

cells generated during the demyelination stage later developed into oligodendrocytes (Godfraind et al., 1989).

In the present study, we demonstrate that the mitotic activity of O-2A lineage cells observed in vivo during demyelination has been maintained in vitro. This proliferation might be mediated by factors active in the culture milieu or by signals experienced in vivo which persist in vitro. The nature of the signal(s) triggering this proliferation is presently unknown. Reactive astrocytes may secrete mitogenic factors while microglia can synthesize lymphokines in response to trauma (Giulian, 1987; Giulian et al., 1988). Since microglial cells are present in our cultures of adult spinal cord and are much more prevalent in cultures of demyelinated tissue, it is possible that O-2A lineage cells proliferate due to factors released into the culture milieu by these cells. In addition to soluble factors, denuded axons or axolemmal components,

which are mitogenic for oligodendrocytes from developing CNS (Chen and DeVries, 1989), may act as mitogens for O-2A lineage cells during demyelination. Denuded axons in demyelinated lesions may stimulate proliferation directly, or may "prime" O-2A lineage cells to divide in response to specific growth factors. Previous studies have shown that oligodendrocytes isolated from adult spinal cord and expressing GC, but not myelin basic protein, can divide in the presence of neurons (Wood and Bunge, 1986). Along with O-2A progenitors, oligodendrocytes expressing GC also proliferated in our cultures from remyelinating animals. Thus, proliferating oligodendrocytes could also be a major source of remyelinating cells. Type 2 astrocytes and cells with a mixed oligodendrocyte-astrocyte phenotype also proliferated in response to demyelination. Cells with characteristics of both oligodendrocyte and astrocyte phenotypes have been described

in several *in vivo* studies of demyelinating tissue (Bunge et al., 1961; Carroll et al., 1987; Godfraind et al., 1989; Raine, 1989). Additionally, ciliary neurotrophic factor, which induces transient GFAP expression in neonatal O-2A progenitors *in vitro*, is much more abundant in regenerating CNS tissue (Nieto-Sampedro et al., 1983) and might possibly induce a mixed phenotype *in vivo*. The presence of phenotypic characteristics of both oligodendrocytes and astrocytes in the same cell indicates that some degree of plasticity is possible between these two differentiation pathways. Mixed phenotype cells might be precursor cells at an intermediate bipotential stage which are responding to two differentiation signals simultaneously, or may be mature cells in a state of trans-differentiation between astrocyte and oligodendrocyte phenotypes. By either mechanism, mixed phenotype cells might provide additional ways to increase the number of oligodendrocytes available for remyelination.

Now that we have established and characterized an *in vitro* system of glial cells derived from normal and demyelinating/remyelinating adult CNS, we can design future experiments to determine which of the proliferating cells provide new oligodendrocytes in remyelination and which factors enhance this pathway. Since we have recently developed a similar *in vitro* system from adult human CNS (Dorn et al., 1990), such experiments could also be performed with human oligodendrocyte lineage cells. The study of the growth and differentiation properties of the glial cell types involved in remyelination may lead to the elaboration of strategies to promote remyelination in human demyelinating diseases.

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