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Pre-Oligodendrocytes from Adult Human CNS

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CNS remyelination and functional recovery often occur after experimental demyelination in adult rodents. This has been attributed to the ability of mature oligodendrocytes and/or their precursor cells to divide and regenerate in response to signals in demyelinating lesions. To determine whether oligodendrocyte precursor cells exist in the adult human CNS, we have cultured white matter from patients undergoing partial temporal lobe resection for intractable epilepsy. These cultures contained a population of process-bearing cells that expressed antigens recognized by the O4 monoclonal antibody, but these cells did not express galactocerebroside (a marker for oligodendrocytes), glial fibrillary acidic protein (a marker for astrocytes), or vimentin. Selective elimination of O4-positive ($O4^+$) cells by complement-mediated lysis resulted in inhibition of oligodendrocyte development *in vitro*. Since $O4^+$ cells have an antigenic phenotype reminiscent of the rat adult oligodendrocyte–type 2 astrocyte progenitor and appear to develop into oligodendrocytes rather than type 2 astrocytes with time in culture, we call them "pre-oligodendrocytes." Neither pre-oligodendrocytes nor oligodendrocytes incorporated ^3H-thymidine in response to rat astrocyte–conditioned medium, platelet-derived growth factor, insulin-like growth factor (IGF-1), or basic fibroblast growth factor (bFGF). However, IGF-1 increased the relative abundance of oligodendrocytes to pre-oligodendrocytes, while bFGF had the opposite effect. Cells with the antigenic phenotype of pre-oligodendrocytes were also identified in tissue prints of adult human white matter. We propose that, in human demyelinating diseases such as multiple sclerosis, pre-oligodendrocytes may divide and/or migrate in response to signals present in demyelinated lesions and thus facilitate remyelination.

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Rapid and efficient neurotransmission is dependent upon the electrical insulating capacity of the myelin sheath around axons (reviewed in Ritchie, 1984a,b). Nerve conduction is impaired after loss of the myelin sheath and results in severe neurological dysfunction in human demyelinating diseases such as multiple sclerosis (MS). Remyelination can occur in the CNS of MS patients but appears to be limited (Perier and Gregoire, 1965; Prineas et al., 1984). Studies of acute MS cases have revealed that recent demyelinating lesions can exhibit remyelination that appears to correlate with the generation of new oligodendrocytes (Prineas et al., 1984; Raine et al., 1988). However, the origin of these new myelin-forming cells is still debated. One possibility is that the adult CNS contains a pool of oligodendrocyte precursor cells; alternatively, surviving oligodendrocytes may proliferate or may even have the ability to dedifferentiate and initiate a new myelination program. Both of these cell types may react to specific signals present during demyelination. It is our goal to understand these cellular and molecular events and ultimately to design ways to enhance remyelination in adult human CNS.

The growth and differentiation of oligodendrocytes has been studied extensively in the developing rodent CNS. *In vitro* analyses have identified precursor cells that give rise to oligodendrocytes (reviewed in Richardson et al., 1990; Dubois-Dalcq and Armstrong, 1992). A rapidly proliferating progenitor cell is recognized by antibodies that bind to gangliosides on the cell surface (such as A2B5 and anti-GD3), and by expression of vimentin, an intermediate-filament protein. As this early progenitor matures, it proliferates more slowly and acquires antigens recognized by the O4 monoclonal antibody. Such O4-positive ($O4^+$) intermediate precursors (Dubois-Dalcq, 1987) or proligodendrocytes (Gard and Pfeiffer, 1989) differentiate into oligodendrocytes that express galactocerebroside (GC) and later turn on myelin protein genes (Dubois-Dalcq et al., 1986). The early progenitors can give rise not only to oligodendrocytes but also to type 2 astrocytes, depending upon the culture conditions. Therefore, this cell type is called the oligodendrocyte–type 2 astrocyte progenitor (O-2A) and this lineage is called the O-2A lineage (Raff et al., 1983). Type 2 astrocytes emerge later in development than type 1 astrocytes, and their function and localization *in vivo* are not clear. Type 1 astrocytes arise during late embryonic life, from a lineage distinct from the O-2A lineage, and secrete growth factors that influence the growth and fate of O-2A lineage cells (reviewed in Richardson et al., 1990; Dubois-Dalcq and Armstrong, 1992). Platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), and basic fibroblast growth factor (bFGF) can each act as mitogens for

O-2A lineage cells. In addition, these growth factors can regulate differentiation within the O-2A lineage. PDGF allows timely differentiation *in vitro* (Raff et al., 1988). IGF-1 induces progenitors to become committed to the oligodendrocyte differentiation pathway (McMorris et al., 1986; McMorris and Dubois-Dalcq, 1988). bFGF can inhibit oligodendrocyte differentiation and myelin gene expression (McKinnon et al., 1990). bFGF in combination with PDGF also promotes continuous proliferation in the absence of differentiation (Bögler et al., 1990).

In vitro studies have shown that neonatal O-2A progenitors can give rise to an adult form of O-2A progenitor in cultures of rat optic nerve (Wolswijk et al., 1990). The adult O-2A progenitor simultaneously expresses antigens recognized by A2B5 and O4 antibodies but does not express vimentin (Wolswijk and Noble, 1989). These cells also have the ability to proliferate and differentiate into oligodendrocytes or type 2 astrocytes *in vitro,* although these events occur more slowly than with the neonatal cells (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; Hunter and Bottenstein, 1991). Analyses of semithin frozen sections and dissociated cell suspensions indicate that cells with the antigenic phenotype of O-2A progenitors probably comprise less than 2% of the cells in adult rodent CNS tissues (Miller et al., 1985; Wood and Bunge, 1991). Only minimal turnover of oligodendrocytes occurs in normal adult rodent CNS (McCarthy and Leblond, 1988). However, new oligodendrocytes are generated during remyelination after a demyelinating episode in the rodent CNS (Herndon et al., 1977; Ludwin, 1979; Aranella and Herndon, 1984; Godfraind et al., 1989). Both O-2A progenitors expressing O4 antigens and oligodendrocytes expressing GC are capable of proliferating in response to signals present during demyelination induced by a coronavirus infection of mice (Godfraind et al., 1989; Armstrong et al., 1990a). Yet the respective contribution of O-2A progenitors and oligodendrocytes in these regeneration events is still unclear (discussed in Dubois-Dalcq and Armstrong, 1990).

Previous studies have shown that oligodendrocytes from adult human brain can be grown in culture (Kim et al., 1983; Kim, 1990). The present *in vitro* and tissue print analyses of adult human white matter demonstrate the existence of cells with the antigenic phenotype characteristic of O-2A progenitors from adult rodent CNS. In tissue culture, these cells do not respond to growth factors that trigger the mitosis of neonatal rodent O-2A progenitors but they appear to develop into oligodendrocytes and are therefore called "pre-oligodendrocytes."

Materials and Methods

Glial cell isolation. Fresh human temporal lobe tissue was obtained from biopsies of patients undergoing therapeutic resection for intractable epilepsy. This tissue does not include the epileptic focus and, when examined histologically, shows a normal cytoarchitecture or mild gliosis. Upon excision, the tissue was immersed in Dulbecco's modified Eagle medium (DMEM; GIBCO, Grand Island, NY) buffered with 1 mM HEPES (GIBCO) and containing 50 μg/ml of gentamycin (Whittaker, Walkersville, MD), 50 U/ml of penicillin, and 50 μg/ml of streptomycin (GIBCO). Meninges and blood vessels were carefully removed, and the majority of gray matter was separated from the white matter, which was then minced finely. Enzymatic and mechanical dissociation of the minced white matter was then performed by a procedure modified from Miller et al. (1985) and Armstrong et al. (1990a). The tissue was incubated at 37°C with sequential enzyme treatments (twice for 20 min in solution 1, and then once for 15 min in solution 2). Solution 1 contained 0.125% trypsin (GIBCO), 20 U/ml papain (Worthington, Freehold, NJ), 0.02% collagenase type III (Worthington), and 20 μg/ml DNase I (Sigma, St. Louis, MO) in minimum essential medium with 25 mM HEPES buffer (MEM-HEPES; GIBCO). Solution 2 contained 0.05% trypsin, 0.02% EDTA (GIBCO); 0.02% collagenase type III; and 20 μg/ml DNase I in MEM-HEPES. The enzymatically digested tissue was then bathed in a third solution [0.25% soybean trypsin inhibitor, 2 μg/ml DNase I, 0.166% BSA (all from Sigma), and 5% fetal bovine serum (FBS; Hyclone, Logan, UT) in MEM-HEPES] and mechanically dissociated through pipettes of decreasing bore diameter (5 ml, 10–20 times; 1 ml, 10–20 times; Pasteur pipette, 10–20 times). Floating cells were transferred to a 50 ml tube that was then filled with DMEM and spun for 10 min at 1500 rpm in a GLC-2B centrifuge (Sorvall, Newton, CT). The supernatant was removed, and the pelleted cells were resuspended in DMEM containing 10% FBS (approximately 8 ml/gm of total tissue). Cell suspension was plated as a drop (200 μl) into each 35 mm plastic dish, which was precoated with 5 μg/ml of poly-D-lysine (Sigma). After incubating these drops for 1 hr at 37°C to enhance cell attachment, 2 ml of DMEM containing 10% FBS were added to each dish.

After 2 d *in vitro,* the cells still floating in the cultures were collected and reseeded (Kim, 1990) onto newly poly-D-lysine–coated 35 mm dishes at a density double that of the initial seeding. This reseeding results in enrichment of cells of the oligodendrocyte lineage. The reseeded cells were fed either with 5% FBS in DMEM or with 1% FBS in a defined medium [DMEM + 5 μg/ml insulin, 50 μg/ml transferrin, 30 nM selenium, 30 nM triiodothyronine (all from Sigma), 1 mM sodium pyruvate (GIBCO), and 25 μg/ml gentamycin; modified from Eccleston and Silberberg (1984)]. In some experiments, this defined medium was conditioned for 2 d by neonatal rat cortical type 1–like astrocytes, prepared as described in Armstrong et al. (1990b). After various times *in vitro* (see Results), cultures were fixed with 2% paraformaldehyde in MEM-HEPES for 15 min.

Immunocytochemistry. O-2A lineage cells were identified by three-color immunofluorescence using the O4 monoclonal antibody, Ranscht anti-GC monoclonal antibody (Ranscht mAb), and a polyclonal antibody against glial fibrillary acidic protein (GFAP). O4 is a mouse monoclonal IgM (Sommer and Schachner, 1981), kindly provided by I. Sommer (Southern General Hospital, Glasgow, Scotland), which recognizes sulfatide, seminolipid, and an unidentified antigen (Bansal et al., 1989). O4 antibody was visualized with rhodamine-conjugated goat anti-mouse IgM (Jackson Immunoresearch Laboratories, West Grove, PA). Ranscht mAb is a mouse IgG3, kindly provided by B. Ranscht (La Jolla Cancer Research Center, La Jolla, CA), which recognizes GC and sulfatide as well as an earlier antigen emerging on the rodent oligodendrocyte surface shortly before the appearance of GC immunostaining (Ranscht et al., 1982; Bansal et al., 1989). This antibody was visualized with fluorescein-conjugated goat anti-mouse IgG3 (Fischer Biotech, Orangeburg, NY). (Without a glycolipid analysis of human glial cell populations in our cultures, we can only assume that the antigens recognized by the O4 and GC antibodies are the same in rodent and human.) The rabbit polyclonal anti-GFAP, kindly provided by R. Pruss (Merrill Dow Pharmaceuticals, Cincinnati, OH), immunostains GFAP but does not react with other intermediate-filament proteins (Pruss et al., 1979). Rabbit anti-GFAP was visualized with biotinylated donkey anti-rabbit IgG (Amersham Chemical Co., Arlington Heights, IL) followed by streptavidin-conjugated 7-amino-4-methyl-coumarin-3-acetic acid (AMCA; Molecular Probes, Inc., Eugene, OR).

Fixed cells were stained simultaneously with O4 and Ranscht mAb, and then the cells were treated with 5% glacial acetic acid in ethanol and stained with anti-GFAP (Armstrong et al., 1990a). For each antigenic phenotype of the O-2A lineage (see Results), cells within every third row of the optical fields visualized with a 25× objective were counted. The sum was multiplied by 3 to obtain an estimate of the total number of cells of each phenotype per 35 mm dish. We note that if the O4 antibody was deleted from the triple-stain procedure, we noticed that more cells stained with the Ranscht mAb antibody than when both antibodies were mixed together. This may be due to the overlapping specificities of these two antibodies (see above), which may be competing for some similar epitope(s), possibly on sulfatides (see above). However, for simplicity, cells stained with the Ranscht mAb will be referred to as "GC+."

In some experiments, a fourth antibody, anti-vimentin, was added to the triple-label procedure described above. This anti-vimentin IgG1 mouse monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) was visualized with fluorescein-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories). Although Ranscht mAb and anti-vimentin antibodies were simultaneously visualized in the fluorescein

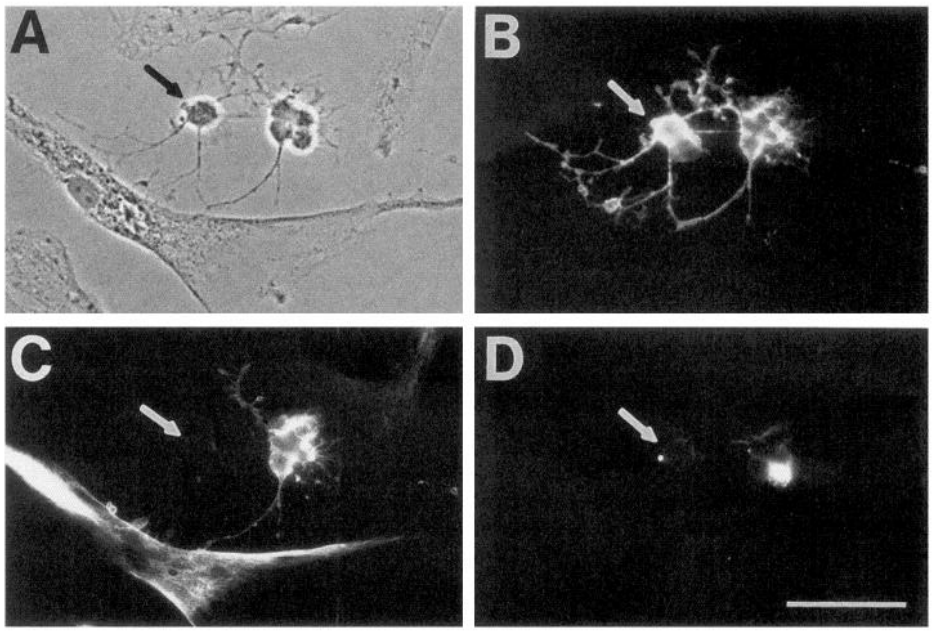

Figure 1. Antigenic phenotype of pre-oligodendrocytes. A typical pre-oligodendrocyte (*arrow*) with its process-bearing morphology is shown by phase contrast (*A*) in a culture grown for 12 d in defined medium with 1% FBS. The same pre-oligodendrocyte expresses surface antigens recognized by O4 (*B*; rhodamine mode) but does not express GC on the cell surface, in contrast to the cluster of oligodendrocytes on the right (*C*; fluorescein mode). This pre-oligodendrocyte does not contain vimentin (also detected in the fluorescein mode in *C*) or GFAP (*D*; coumarin mode), although intracellular vimentin is evident in the fibroblastic cell to the lower left in *C*. Scale bar, 50 μm.

channel, they were easily distinguished since Ranscht mAb immunostained the cell surface while anti-vimentin immunostained intracellular filaments. Finally, in a few cases, the triple-label protocol was modified to examine expression of other antigens: the O4 IgM primary antibody was then replaced with either A2B5 or LB1. A2B5 and LB1 (anti-GD3) are both IgM monoclonal antibodies that recognize gangliosides (Eisenbarth et al., 1979; Curtis et al., 1987). All immunostained dishes were visualized and photographed using a Zeiss Axiophot or Photoscope III microscope equipped with appropriate filters for triple immunofluorescence.

Complement-mediated cell lysis. Complement-mediated lysis of specific cell populations was achieved by first incubating cultures of living cells for 1 hr at 37°C with either O4 or Ranscht mAb, diluted 1:6 or 1:2, respectively, in defined medium (see above) containing 1% FBS. The cultures were then rinsed twice in DMEM to remove excess antibody and treated for 1 hr at 37°C with rabbit complement (Cedar Lane, Hornby, Ontario, Canada) diluted 1:8 in defined medium (see above) containing 1% FBS. Cultures were rinsed twice in DMEM, fed defined medium containing 1% FBS, and fixed 1 hr or 1 week after complement treatment. Both O4 and Ranscht mAb can bind to myelin debris, which could compete with antibody binding sites on the cell surface and render complement-mediated cell lysis less efficient. Therefore, in these cytolysis experiments, we used primary cultures, which contain less myelin debris than reseeded cultures at early times *in vitro* because the myelin debris is transferred along with the floating cells at the reseeding step (see above).

^{3}H-thymidine labeling and autoradiography. After 2, 6, 13, 20, or 27 d *in vitro* methyl-^{3}H-thymidine (67 Ci/mmol; New England Nuclear, Boston, MA) was added (0.05 μCi/ml culture medium) for 72 hr to cultures grown either in DMEM with 5% FBS or in defined medium (see above) conditioned by rat cortical astrocytes and supplemented with 1% FBS. These cultured cells were then fixed and immunostained as described above. The cultures were then dehydrated in graded alcohols (50%, 75%, 95%; 30 sec in each), air dried, coated with Kodak NTB2 nuclear track emulsion (diluted 1:2), exposed at 4°C for 5 d, developed at 16°C in Kodak D19, and fixed with Kodak fixer.

Growth factors. Human platelet-derived growth factor (20 ng/ml; A and B chain heterodimer from R & D Systems, Minneapolis, MN), human insulin-like growth factor 1 (100 ng/ml; Amgen Biologicals, Thousand Oaks, CA), and bovine basic fibroblast growth factor (20 ng/ml; R & D Systems) were added to the culture dishes after the reseeding step at 2 d. The effects of growth factors were assayed in defined medium containing 50 ng/ml insulin (rather than 5 μg/ml as described above) and 1% FBS. At 4 and 6 d, the growth factors were replenished in each culture. Mitogenic activity of the growth factors was tested by adding ^{3}H-thymidine (see above) to the cultures at 6 d and fixing at 9 d before triple labeling for O4, GC, and GFAP as described above. O-2A lineage cells were counted in at least 50 fields (25× objective) of each dish, and the percentage of cells with each antigenic phenotype within the total O-2A lineage population was calculated.

Tissue print. A "touch preparation" of fresh human CNS was prepared by a tissue printing technique modified from Barres et al. (1990). Temporal lobe white matter was dissected, cut into thin slices, and digested at 37°C for 45 min in enzymes [30 U/ml papain (Worthington), 0.02% collagenase type III, and 20 μg/ml DNase I in MEM-HEPES with 0.2 mM EDTA (Sigma) and 1 mM cysteine (Sigma)]. The digested tissue was transferred to a solution containing 0.2% ovomucoid (Worthington), 2 μg/ml DNase I, and 0.166% BSA for 5 min and then transferred to 0.2 M $CaCl_2$ (Sigma). Tissue slices were placed onto pieces of nitrocellulose (Schleicher & Schuell, Keene, NH) for support and then touched gently onto coverslips that were precoated with Cell-Tak (Biopolymers, Inc., Farmington, CT) and prerinsed in 0.2 M $CaCl_2$. The tissue adhering to each coverslip was immediately fixed with 2% paraformaldehyde in MEM-HEPES for 15 min and subsequently immunostained as described above except that nuclei were also stained with 4′,5-diamidino-2-phenylindole (DAPI; 100 μg/ml; Sigma). DAPI and anti-GFAP antibody were simultaneously visualized in the coumarin channel but were easily distinguished since DAPI was localized in nuclei and anti-GFAP reacted with intermediate filaments in the cytoplasm. The abundance of pre-oligodendrocytes was determined by counting the number of pre-oligodendrocytes identified while counting 100 oligodendrocytes per coverslip. This method ensured that the sample population was composed of sufficiently dispersed and appropriately immunostained tissue.

Results

Antigenic characterization and differentiation potential of pre-oligodendrocytes in vitro

The glial cell population in cultures of adult human white matter was first characterized after 1–2 weeks of growth in defined medium supplemented with 1% FBS. Three-color immunofluorescence (rhodamine, fluorescein, and AMCA fluorochromes) was used to visualize three antigens simultaneously. Oligodendrocytes were identified by the expression of GC (Raff et al., 1978), while astrocytes were identified by expression of GFAP (Bignami et al., 1972; Raff et al., 1979). A significant population of process-bearing cells was stained by O4 antibody but not by Ranscht mAb (which binds to GC; see Materials and Methods) or an anti-GFAP antibody (Fig. 1). These cells did not express

vimentin, an intermediate-filament protein (Fig. 1) and therefore closely resemble O-2A progenitors from adult rodent optic nerve (Wolswijk and Noble, 1989). However, these human O4$^+$ cells did not stain with two monoclonal antibodies, A2B5 and LB1, that recognize gangliosides on the surface of rodent O-2A progenitor cells (Raff et al., 1983; Levi et al., 1987), possibly because of differences between rodent and human CNS ganglioside epitopes (data not shown).

We then analyzed how the proportion of the different glial cell phenotypes evolves with time in culture using triple immunofluorescence. With this technique, we can identify four populations of cells within the O-2A lineage: (1) putative progenitor cells (O4$^+$ GC$^-$ GFAP$^-$), (2) oligodendrocytes (O4$^+$ GC$^+$ GFAP$^-$), (3) type 2 astrocytes (O4$^+$ GC$^-$ GFAP$^+$) (Trotter and Schachner, 1989), and (4) mixed oligodendrocyte-astrocyte phenotype cells (O4$^+$ GC$^+$ GFAP$^+$). O-2A lineage cells expressing GFAP (type 2 astrocytes or mixed phenotype cells) were infrequent in cultures grown in medium with low serum (Fig. 2), and their number was only slightly higher in cultures grown in DMEM with 5% FBS (data not shown). The cells expressing only O4 antigens were most abundant at 1 week *in vitro*, constituting almost 50% of the O-2A lineage population, but their number was considerably decreased at 2 weeks and even more so at 3 weeks (Fig. 2). In contrast, the number of oligodendrocytes usually increased between 1 and 3 weeks in culture (Fig. 2). This shift in the relative ratio of glial cell populations was observed in cultures of three biopsies in several media (DMEM with 5% FBS, defined medium with 1% FBS, or defined medium conditioned by rat cortical astrocytes). This cell population analysis suggests that O4$^+$ GC$^-$ GFAP$^-$ cells can evolve into oligodendrocytes and therefore can be called "pre-oligodendrocytes."

In order to demonstrate directly that pre-oligodendrocytes can become oligodendrocytes in our cultures, we attempted to deplete the pre-oligodendrocyte (O4$^+$-only) and/or the oligodendrocyte (O4$^+$ GC$^+$) population by complement-mediated lysis (Fig. 3). (For efficient complement-mediated lysis, we had to use primary cultures that contain less myelin debris, but also fewer pre-oligodendrocytes relative to oligodendrocytes than the reseeded cultures; compare Fig. 3, upper left, with Fig. 2.) In cultures treated at 5 d with rabbit complement alone (diluted 1:8), the number of O4$^+$-only or O4$^+$ GC$^+$ cells was not markedly reduced relative to nontreated cultures (Fig. 3) even at complement dilutions of 1:4 and 1:2 (data not shown). As predicted, the number of O4$^+$-only cells started to decrease during the next week while the number of O4$^+$ GC$^+$ cells began to increase in both nontreated and complement-treated cultures (by 1.3-fold and 1.2-fold, respectively). In cultures treated with Ranscht mAb and complement, lysis of O4$^+$ GC$^+$ but not O4$^+$-only cells occurred within 1 hr after treatment (Fig. 3). As in control conditions, during the following week *in vitro* the number of O4$^+$-only cells decreased while the number of O4$^+$ GC$^+$ cells increased 2.5 times. In parallel cultures incubated with O4 antibody and complement, both the O4$^+$-only and the O4$^+$ GC$^+$ populations were severely reduced in number 1 hr after treatment (Fig. 3). During the subsequent week *in vitro*, the number of O4$^+$ GC$^+$ cells did not increase. Thus, an increase in oligodendrocytes with time in culture occurred only when the O4$^+$-only population was not drastically depleted early on, reinforcing the concept that these cells develop into oligodendrocytes.

It has been reported that O-2A progenitors and oligodendrocytes from adult rat CNS are susceptible to antibody-independent lysis by rabbit complement (Wren and Noble, 1989). Such an effect was not seen with human glial cells, perhaps because of a difference in the activity of rabbit complement for rodent versus human target cells, or because our cultures were treated with complement later *in vitro* (5 d) than in the rodent study (3 d).

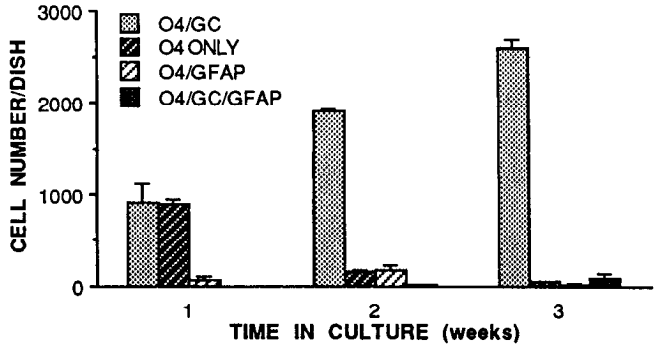

Figure 2. Over time in culture, the number of pre-oligodendrocytes decreases while the number of oligodendrocytes increases. After 1 week *in vitro*, the numbers of oligodendrocytes (*O4/GC*) and pre-oligodendrocytes (*O4 ONLY*) were approximately equal. By 2 weeks, the number of oligodendrocytes has almost doubled, and after 3 weeks, pre-oligodendrocytes had almost disappeared from the cultures while the oligodendrocyte population had further increased. Type 2 astrocytes (*O4/GFAP*) and mixed oligodendrocyte–astrocyte phenotype cells (*O4/GC/GFAP*) were rare at all times examined. All cultures were grown in defined medium conditioned by rat cortical astrocytes and supplemented with 1% FBS. The values shown are averages (±SE) of triplicate dishes for each time point. This is one analysis of a representative set of reseeded cultures prepared from the same biopsy specimen. Similar results were obtained from two other biopsies, but data from separate biopsies were not combined because the cell yield varied markedly.

Lack of evidence of DNA synthesis in pre-oligodendrocytes

To explore the possibility that pre-oligodendrocytes divide before differentiating, we treated the cultures for 72 hr with ^{3}H-thymidine at various times *in vitro*, as shown in Figure 4. Cultures were then processed for three-color immunofluorescence combined with ^{3}H-thymidine autoradiography. Regardless of the growth media used (DMEM with 5% FBS or defined medium conditioned by rat cortical astrocytes and supplemented with 1% FBS) or when the ^{3}H-thymidine was administered, ^{3}H-thymidine incorporation into pre-oligodendrocytes or oligodendrocytes was never detected. In contrast, up to 40% of type 1 astrocytes showed DNA synthesis (Fig. 5). These experiments were repeated with tissue from three separate biopsies and gave identical results.

Since PDGF, IGF-1, and bFGF can each induce proliferation of rat neonatal O-2A progenitor cells (reviewed in Richardson et al., 1990; Dubois-Dalcq and Armstrong, 1992), we tested whether these growth factors would trigger DNA synthesis in the adult human pre-oligodendrocytes or oligodendrocytes in our cultures. Cultures were treated with each growth factor from 2 to 9 d and exposed to a pulse of ^{3}H-thymidine from 6 to 9 d (Fig. 4, third time line). None of these growth factors induced ^{3}H-thymidine incorporation into pre-oligodendrocytes or oligodendrocytes (PDGF, $n = 4$, combined from two biopsies; IGF-1, $n = 2$, one biopsy; bFGF, $n = 4$, combined from two biopsies). Since bFGF can upregulate expression of PDGF receptors in neonatal O-2A progenitors from developing rodent CNS (McKinnon et al., 1990), some cultures were treated with

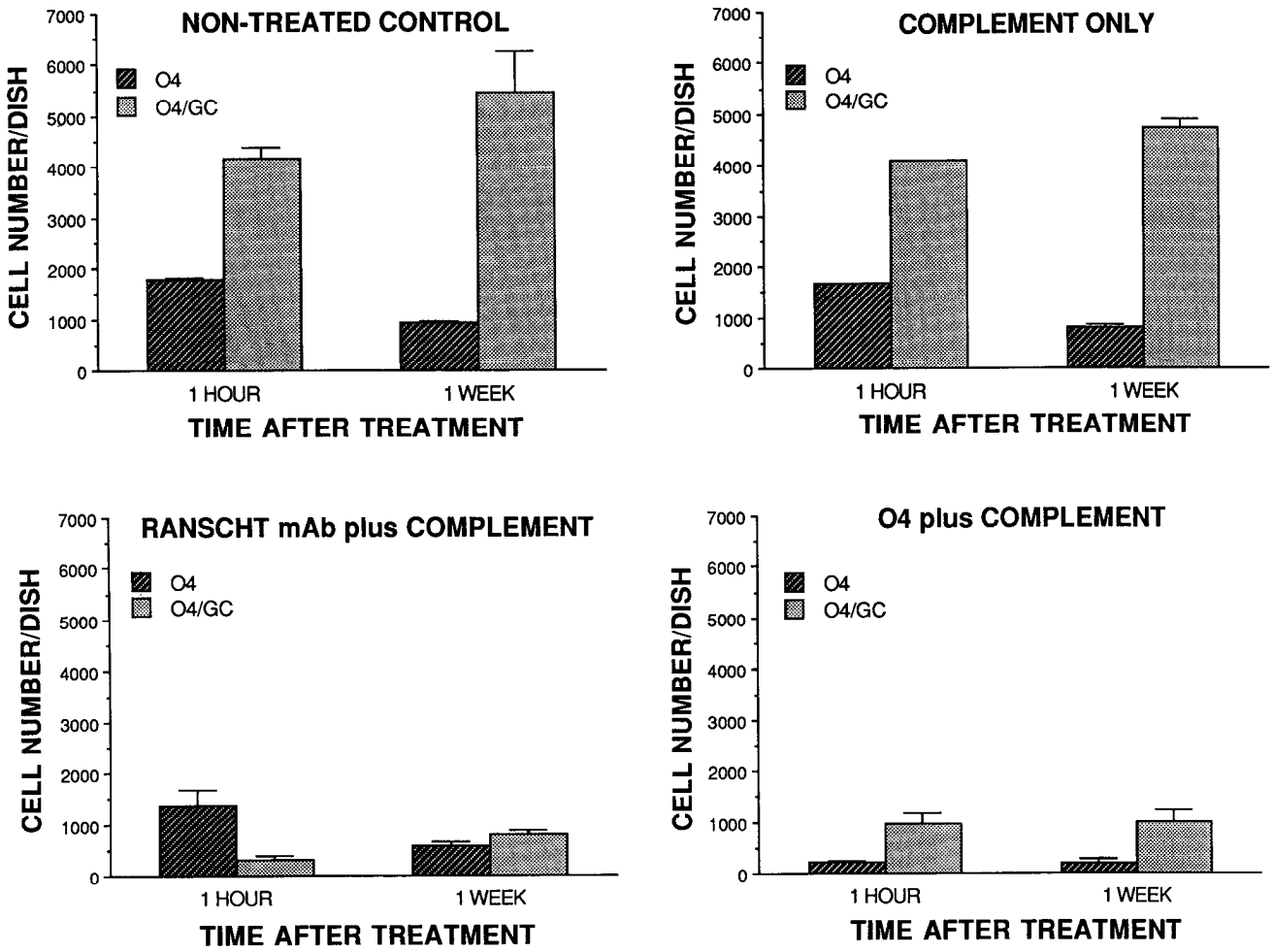

Figure 3. Development of oligodendrocytes with time is inhibited by depletion of the pre-oligodendrocyte population by complement-mediated cell lysis. Primary cultures not treated with complement showed a decrease of pre-oligodendrocytes (*O4*) and increase of oligodendrocytes (*O4/GC*) between 5 and 12 d *in vitro* (corresponding to "1 HOUR" and "1 WEEK" after complement treatment). Cultures treated with rabbit complement alone and fixed either 1 hr or 1 week later contained numbers of cells similar to those of the control. In cultures treated with Ranscht mAb and then complement, the oligodendrocyte population was severely reduced while a substantial population of pre-oligodendrocytes remained. During the subsequent week, the pre-oligodendrocyte cell number decreased while a slight increase in oligodendrocyte number was observed. In cultures treated with O4 antibody and then complement, both the pre-oligodendrocyte and oligodendrocyte populations were severely reduced since most oligodendrocytes express O4. The dramatic reduction of the pre-oligodendrocyte population (compare with upper graphs) prevented an increase of oligodendrocytes during the following week in culture. The values shown are the averages (±SE) of duplicate cultures prepared in parallel from one biopsy (except for the value for 1 hr after complement treatment alone, which is a single sample).

PDGF and bFGF or treated with bFGF from 2 to 4 d followed by PDGF from 4 to 9 d. Our preliminary results from cultures of a single biopsy indicate that these combinations of growth factors also failed to induce ^{3}H-thymidine incorporation into pre-oligodendrocytes or oligodendrocytes.

In vitro *regulation of pre-oligodendrocyte and oligodendrocyte phenotype by bFGF and IGF-1*

Since PDGF, IGF-1, and bFGF can influence differentiation of O-2A progenitors from neonatal rodent CNS, we examined whether these growth factors could modulate the antigenic phenotypes expressed by O-2A lineage cells in our cultures of adult human CNS. In the absence of these growth factors or in the presence of PDGF between 2 and 9 d, pre-oligodendrocytes constituted 53% and 54% of the O-2A lineage population, respectively, in a series of three biopsies (Fig. 6). In contrast, cultures grown in the presence of bFGF contained 78% pre-oligodendrocytes and only 13% oligodendrocytes. IGF-1 treatment decreased the percentage of pre-oligodendrocytes to 43%. These results indicate that IGF-1 favored oligodendrocyte differentiation while bFGF seemed to inhibit and/or reverse this process. Since astrocytes are present in these cultures, this inhibition of differentiation by bFGF could possibly result from cooperation between bFGF and astrocyte-derived PDGF (Bögler et al., 1990). In fact, simultaneous addition of PDGF and bFGF to the cultures resulted in inhibition of differentiation similar to that seen with bFGF alone (data not shown).

Evidence that pre-oligodendrocytes exist in the white matter in vivo

To determine whether the different glial cell antigenic phenotypes characterized *in vitro* also exist in the adult human CNS, we prepared tissue prints, which produce a thin layer of cells adhering to a coverslip. The tissue was subjected to mild enzymatic digestion before being touched to the surface of a coverslip, which was then immediately fixed in paraformaldehyde.

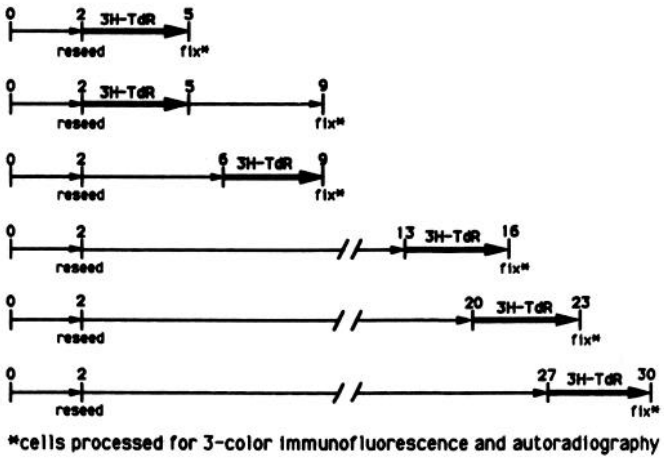

Figure 4. Time courses followed for *in vitro* ^{3}H-thymidine labeling studies. Days *in vitro* are indicated along each time line. All these pulse labeling experiments were performed in cultures grown in DMEM with 5% FBS as well as in those grown in defined medium conditioned by rat cortical astrocytes and supplemented with 1% FBS. ^{3}H-thymidine incorporation into pre-oligodendrocytes or oligodendrocytes was not detected in either growth medium. For each time point and growth medium, triplicate cultures were examined.

Thus, the CNS cells were not subjected to the trauma of mechanical dissociation and culture, which might modify their antigenic phenotypes. In such tissue prints of adult human white matter, oligodendrocytes were easily identified by cell surface expression of GC (Fig. 7). (We did not find cells with intracellular filaments immunoreactive for anti-GFAP in adult human white matter, although they were readily observed in tissue prints of adult rodent gray matter.) Cells with the antigenic phenotype of pre-oligodendrocytes ($O4^+$ GC^- $GFAP^-$) were consistently found in these tissue prints, although relative to oligodendrocytes, they were infrequent (7.6 ± 1.0 pre-oligodendrocytes identified for every 100 oligodendrocytes counted; triplicate coverslips from one biopsy) (Fig. 7). Yet, this preliminary analysis of tissue prints suggests that pre-oligodendrocytes may exist in the adult human white matter.

Discussion

We have found that cultures of adult human white matter contain glial cells that express antigens recognized by the O4 monoclonal antibody but do not express differentiation markers of oligodendrocytes, such as GC. The complement-mediated lysis experiment clearly demonstrates that the antibodies described by Ranscht et al. (1982) and Bansal et al. (1989) can specifically

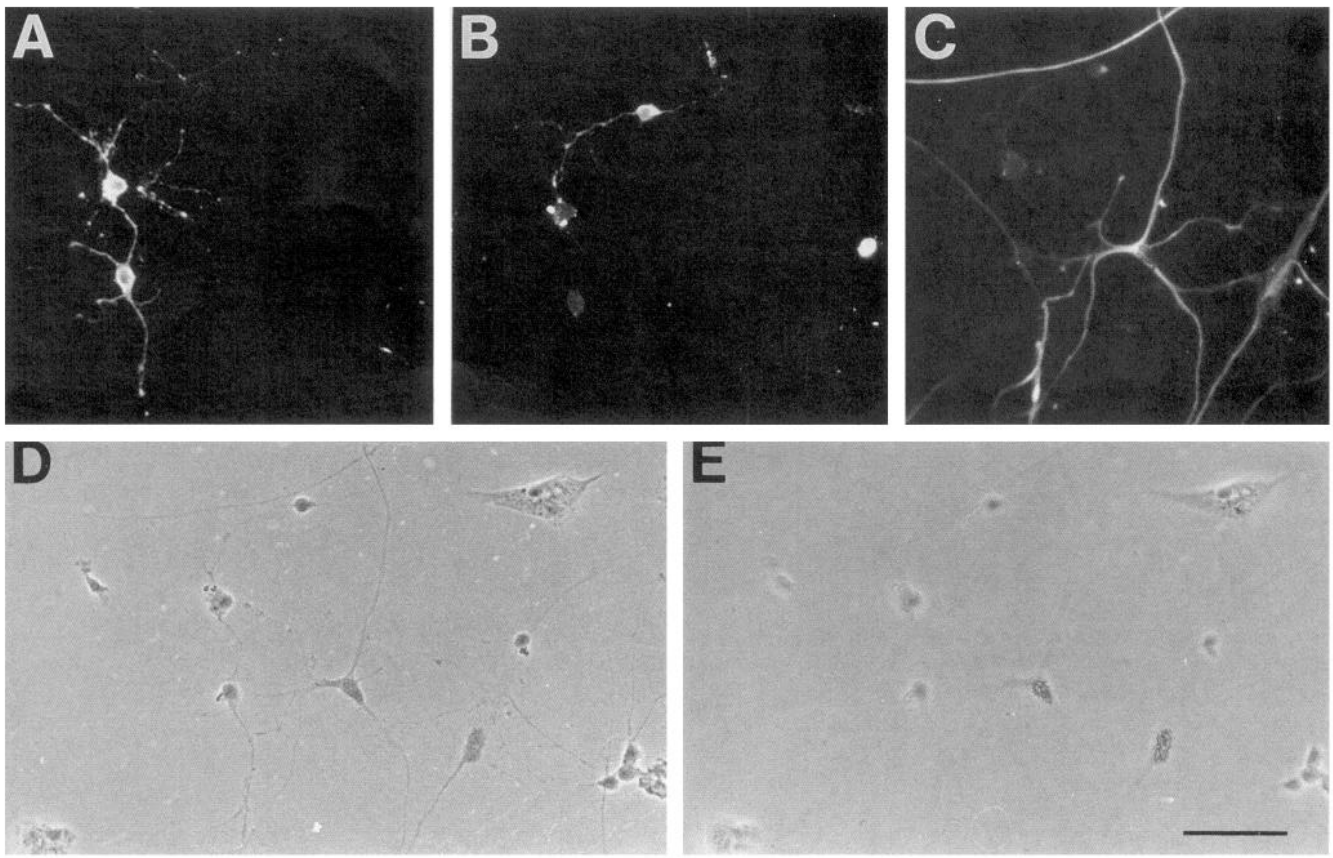

Figure 5. Neither pre-oligodendrocytes nor oligodendrocytes incorporated ^{3}H-thymidine. A pulse labeling experiment was done as shown on the fourth time line of Figure 4. Two pre-oligodendrocytes, on the *left* in each field, express O4 (*A*; rhodamine mode) but not GC (*B*; fluorescein mode) or GFAP (*C*; coumarin mode). An oligodendrocyte in the *upper middle* of the field expresses only GC (*B*). Two type 1 astrocytes, toward the *lower right*, express GFAP (*C*) but not O4 antigens (*A*). The phase-contrast image focused on the cells (*D*) shows the cell nuclei and when focused on the emulsion layer (*E*) shows the silver grains only over the type 1 astrocyte nuclei, indicating ^{3}H-thymidine incorporation. Scale bar, 50 μm.

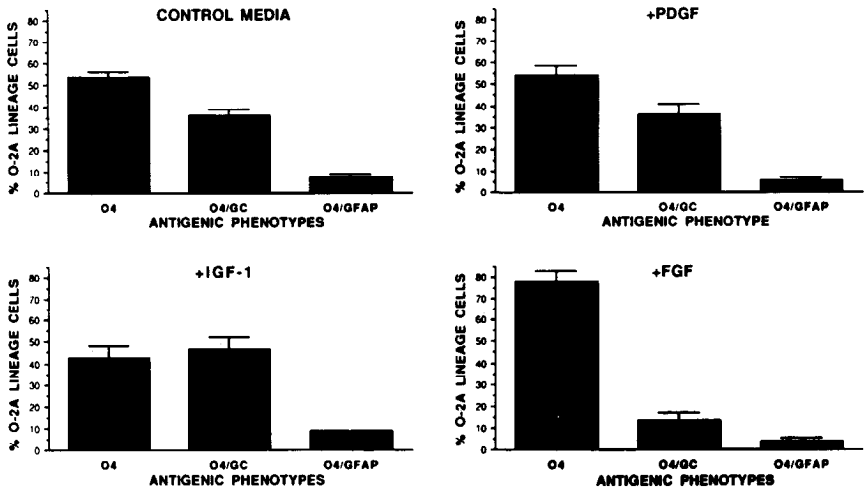

Figure 6. Effects of IGF-1 and bFGF on the pre-oligodendrocyte and oligodendrocyte phenotypes. After 9 d of growth in defined medium with 1% FBS, approximately 53% of the O-2A lineage cells were pre-oligodendrocytes (*O4*), 37% were oligodendrocytes (*O4/GC*), 8% were type 2 astrocytes (*O4/GFAP*), and 2% were mixed phenotype cells (O4/GC/GFAP; not shown). The relative abundance of these cell types was not changed by addition of PDGF to the control medium. The presence of IGF-1, however, resulted in an increased percentage of oligodendrocytes. In contrast, with bFGF treatment the large majority of O-2A lineage cells expressed the pre-oligodendrocyte phenotype. [Ninety-five percent confidence intervals for values from the control cultures (n = 8; combined from three biopsies) extended from 47% to 61% for pre-oligodendrocytes and 29% to 43% for oligodendrocytes. Values (±SE) determined in the presence of PDGF (n = 4; combined from two biopsies) were within these limits while those for IGF-1 (n = 2; one biopsy) and bFGF (n = 4; combined from two biopsies) were outside of these 95% confidence intervals.]

eliminate human cells recognized as GC$^+$ oligodendrocytes by immunostaining while preserving the great majority of the O4$^+$-only cells (Fig. 3). Based on time sequence studies and complement-mediated killing of these O4$^+$-only cells, it appears that they have the potential to develop into oligodendrocytes and therefore can be called "pre-oligodendrocytes." Pre-oligodendrocytes did not divide in response to PDGF, IGF-1, bFGF, or astrocyte-conditioned medium. However, bFGF clearly favored the pre-oligodendrocyte phenotype, while IGF-1 treatment increased expression of the oligodendrocytic phenotype. Moreover, cells with the pre-oligodendrocyte phenotype were identified in tissue prints of adult human white matter, suggesting that these precursors exist in adult human brain *in vivo*. Further experiments are needed to determine if pre-oligodendrocytes represent an early stage of oligodendrocyte differentiation or a true progenitor able to divide in response to appropriate stimuli.

From birth to adult life, the rodent nervous system has been shown to contain precursors of oligodendrocytes (see introductory remarks). Neonatal O-2A progenitors of optic nerve will, after an initial proliferation period driven by type 1 astrocytes *in vitro*, generate slowly proliferating O4$^+$ vimentin$^-$ cells that appear committed to become oligodendrocytes (Dubois-Dalcq, 1987). Similar cells, referred to as "proligodendrocytes," can be isolated from neonatal rodent brain, and the great majority of them (80%) will become oligodendrocytes while a smaller fraction (20%) will remain quiescent and may persist in adult brain (Gard and Pfeiffer, 1989). Interestingly, neonatal O4$^+$ precursor cells divide optimally in response to neuron-derived factors (Gard and Pfeiffer, 1990). The adult rodent optic nerve also yields oligodendrocyte progenitor cells (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). As in cultures of neonatal rat optic nerve, these progenitor cells can differentiate into type 2 astrocytes, when grown in high concentrations of FBS, and are therefore called adult O-2A progenitors. These cells express antigens recognized by O4 antibodies in the absence of GC, GFAP, or vimentin, an antigenic phenotype similar to that of human pre-oligodendrocytes. When cocultured with neonatal rat astrocytes, O-2A progenitors from adult optic nerve proliferate slowly, with an approximately 65 hr cell cycle (Wolswijk and Noble, 1989; Chan et al., 1990). In contrast, human pre-oligodendrocytes do not exhibit proliferation in the conditions tested and appear committed mainly to the oligodendrocyte differentiation pathway. These properties of human pre-oligodendrocytes are similar to those described for O4$^+$ cells isolated from adult rodent spinal cord (Wood and Bunge, 1991). These cells represent only 2% of the rat spinal cord glial cell population and do not proliferate well when cocultured with rat dorsal root ganglion neurons (Wood and Bunge, 1991). Taken together, these observations suggest that pre-oligodendrocytes are rare in normal adult CNS as also indicated by our tissue print analysis of adult human white matter.

The possibility has been raised that adult GC$^+$ oligodendrocytes can dedifferentiate, reenter the mitotic cycle, and later give rise to remyelinating cells (Wood and Bunge, 1991). When a purified fraction of rat adult oligodendrocytes (97% GC$^+$) was cocultured with rat dorsal root ganglion neurons, almost 50% of the cells became O4$^+$ GC$^-$ by 8 d *in vitro* and proliferated (Wood and Bunge, 1991). Thus, a population of adult rodent O4$^+$ GC$^-$ cells developed from O4$^+$ GC$^+$ cells in response to the culture environment, suggesting that dedifferentiation had occurred in the early stages of the culture. Similarly human pre-oligodendrocytes may arise from oligodendrocytes that lose GC expression during the first days *in vitro*. Clearly, further experiments with human glial cells are needed to investigate this possibility.

Dedifferentiation of oligodendrocytes *in vitro* may in fact mimic some of the events occurring during demyelination *in vivo*. Studies of experimental demyelination in adult mouse spinal cord have shown that O4$^+$-only cells and oligodendrocytes increase in number and proliferate in response to demyelination induced by a murine coronavirus and that this proliferation precedes remyelination (Godfraind et al., 1989; Armstrong et al., 1990a).

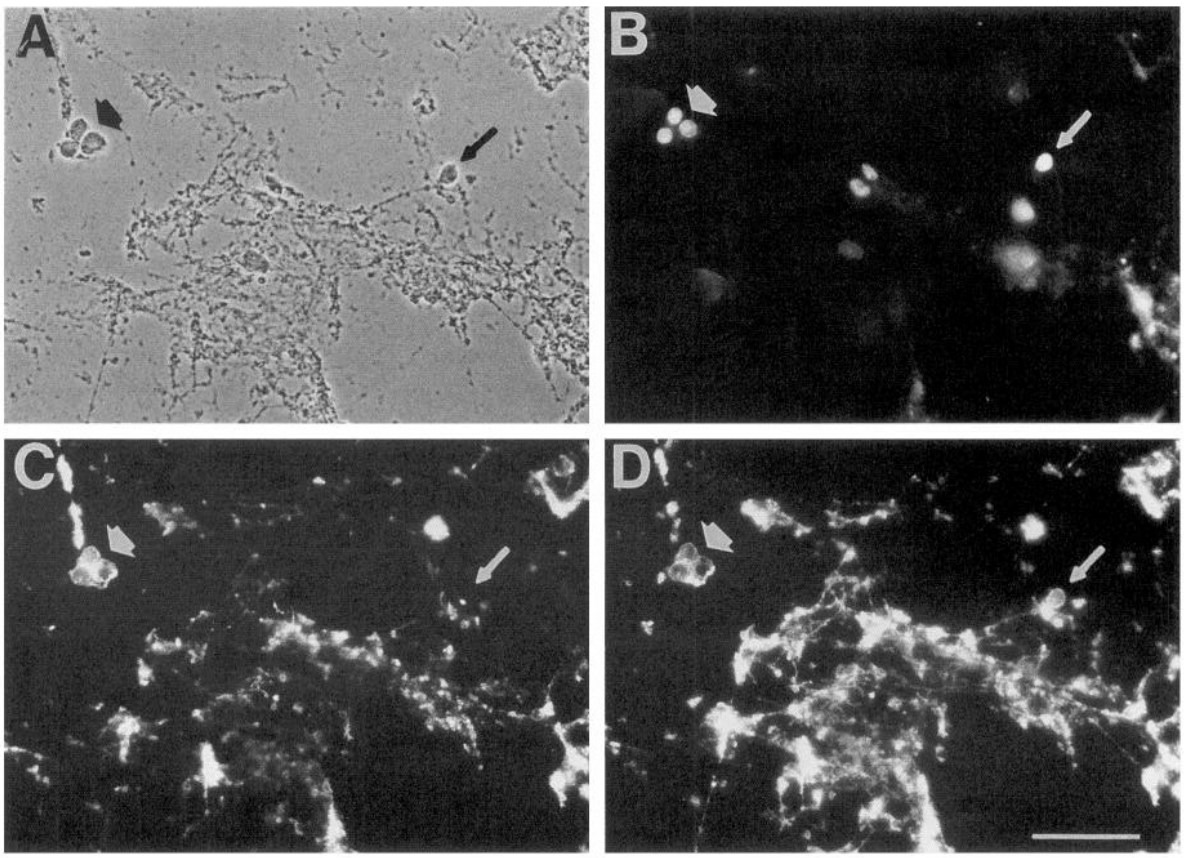

Figure 7. Pre-oligodendrocytes are present in tissue prints of adult human white matter. Phase contrast (*A*) shows the cells and processes of adult human white matter acutely dissociated by the tissue print technique. The nuclei of intact cells are visible with DAPI stain (*B*; coumarin mode). A cluster of three oligodendrocytes (*thick arrow*) binds Ranscht mAb (*C*; fluorescein mode) while a pre-oligodendrocyte (*thin arrow*) does not express GC (*C*) or GFAP (*B*) but expresses O4 antigens (*D*; rhodamine mode). Scale bar, 50 μm.

In addition, O-2A lineage cells cultured from this demyelinated mouse spinal cord tissue respond to IGF-1 by increased expression of the oligodendrocyte phenotype (Armstrong et al., 1990a) as was observed in cultures of normal adult human white matter. Interestingly, treatment with bFGF increased the proportion of type 2 astrocytes ($O4^+$ $GFAP^+$) in cultures from demyelinated mouse tissue, whereas bFGF increased the abundance of pre-oligodendrocytes in our cultures of normal human brain. This difference may be due to the presence of signals specific to the demyelinated tissue, which may induce GFAP expression in $O4^+$ GC^- cells. These studies demonstrate that $O4^+$-only cells may divide in response to demyelination and may contribute, together with oligodendrocytes, to the efficient remyelination process in rodents (Herndon et al., 1977; Ludwin, 1979; Aranella and Herndon, 1984; Godfraind et al., 1989; Armstrong et al., 1990a).

The role of pre-oligodendrocytes in CNS remyelination in human remains to be determined. To understand mechanisms of myelin repair in demyelinating diseases such as MS, we need to elucidate the nature of the signals for mitosis, migration, and/or differentiation of adult pre-oligodendrocytes and oligodendrocytes. Since astrocyte-conditioned medium is mitogenic for O-2A progenitors from adult rat CNS, astrocyte-derived factors may act as mitogens in demyelinated tissue (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; Chan et al., 1990). A recent study has demonstrated that PDGF can induce mitosis of O-2A progenitors from adult rat optic nerve (Wolswijk et al., 1991). Similarly, coculturing with rat neurons and growth in conditioned medium from a rat CNS neuronal cell line enhance division of O-2A progenitors and/or oligodendrocytes from adult rodent CNS (Wood and Bunge, 1986, 1991; Hunter and Bottenstein, 1991), suggesting that neuronal signals may stimulate proliferation in demyelinated lesions. Defined growth factors have not yet been identified as mitogens for pre-oligodendrocytes or oligodendrocytes from human CNS (Yong et al., 1988; present results). We have examined the potential effects of neuron-derived mitogens by culturing adult human glial cells with bovine axolemma-enriched fraction (Detskey et al., 1987) or the HCN-1 human neuronal cell line (Ronnett et al., 1990). In these preliminary studies, neither pre-oligodendrocytes nor oligodendrocytes incorporated ^{3}H-thymidine that was added to the medium from 6 to 9 d *in vitro* (R. C. Armstrong, C. V. Kufta, E. Friedman, and M. E. Dubois-Dalcq, unpublished observations). It may be that interaction with intact differentiated neurons and/or their processes is necessary to stimulate mitosis of human oligodendrocytes and/or pre-oligodendrocytes. In MS tissue, oligodendroglial hyperplasia at the periphery of demyelinating lesions has been observed in association with remyelination (Raine and Scheinberg, 1988; Raine et al., 1988). Thus, oligodendrocytes appear to be generated in adult human CNS in response to signals present in demyelinating lesions. In addition, migration of myelin-forming cells and/or their precursors

may be a prerequisite for remyelination of large lesions. *In vitro* studies may help determine the migratory response of pre-oligodendrocytes and/or oligodendrocytes to signals present in MS plaques.

In conclusion, the present study extends the analyses of oligodendrocyte precursor cells in the adult CNS from rodent to human. Rare pre-oligodendrocytes can be detected in tissue prints of normal adult human white matter. In the early stages of culture, this population of cells appears amplified, possibly because of oligodendrocyte dedifferentiation. With time in culture, however, it appears that the ultimate fate of these cells is to become oligodendrocytes and that this process is modulated by certain growth factors. Our *in vitro* system should allow characterization of the signals that may activate these pre-oligodendrocytes and guide them during remyelination *in vivo*.

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