INTRODUCTION

It is not known how the many different types of neurons and glia in the mature central nervous system (CNS) are generated from the neuroepithelial cells that line the lumen of the neural tube. We are addressing this question by focussing on the development of oligodendrocytes, the myelinating cells of the CNS, from their neuroepithelial precursors in the embryonic rat spinal cord. We recently presented evidence in support of the idea that the ventricular zone (VZ) of the neural tube is a mosaic of specialized neural precursors that express different sets of gene rec
suggested might be devoted specifically to the production of oligodendrocyte progenitors (Pringle and Richardson, 1993; Yu et al., 1994). This specialized microdomain comprises a narrow, longitudinal ribbon of neuroepithelial cells that can be recognized in situ by several molecular markers characteristic of the oligodendrocyte lineage: 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP; Yu et al., 1994), antigens recognized by monoclonal antibody O4 (Ono et al., 1995), the platelet-derived growth factor alpha-receptor (PDGFRα; Pringle and Richardson, 1993; Yu et al., 1994; Nishiyama et al., 1996) and, possibly, the myelin proteolipid protein or a related isoform (PLP/DM-20; Timsit et al., 1995). Of these, PDGFRα seems to be a general marker, being expressed at an equivalent developmental stage and location in the VZ of the rat, mouse, chicken and *Xenopus* spinal cords (Pringle and Richardson, 1993; Pringle et al., 1996; N. Pringle, unpublished). After their first appearance at the ventricular surface of the E14 rat spinal cord, the PDGFRα

$^{+}$ cells seem to proliferate and migrate away from the VZ, becoming widely distributed throughout the cross section of the cord and reaching the dorsal-most regions between E16 and E18 (Pringle and Richardson, 1993).

We describe experiments designed to test the idea that these PDGFRα^{+} cells are oligodendrocyte precursors, and to determine the contribution they make to oligodendrogenesis in the spinal cord. We purified PDGFRα^{+} cells from embryonic rat spinal cord by immunoselection and found that they all differentiate into oligodendrocytes when cultured under appropriate conditions in vitro. Very few oligodendrocytes developed in cultures of embryonic spinal cord cells that had been depleted of PDGFRα^{+} precursors by antibody-mediated complement lysis. When dorsal and ventral E14 rat spinal cord cells were cultured separately, only ventral cultures contained PDGFRα^{+} precursors and gave rise to differentiated oligodendrocytes. The ability of dorsal cells to generate oligodendrocytes was acquired after E16 in parallel with the appearance of PDGFRα^{+} cells. These data demonstrate that PDGFR

MATERIALS AND METHODS

Optic nerve cell cultures

The optic nerves of newborn or postnatal day 7 (P7; the day of birth is P0) Sprague-Dawley rat pups were dissected and dissociated as described previously (Miller et al., 1985). Approximately 2,000 cells were seeded in a 10 μl droplet on a 13 mm diameter poly-D-lysine-coated glass coverslip in modified Bottenstein and Sato's (BS) medium (Bottenstein and Sato, 1979) containing 10% FCS. After 30 minutes, 400 μl BS medium was added to dilute the FCS to 0.5%. Sometimes 10 ng/ml recombinant human PDGF-AA (Peprotech, New Jersey, USA) was added. BS medium is Dulbecco's modified Eagle's medium (DMEM) supplemented with transferrin (0.1 mg/ml), bovine serum albumin (0.1 mg/ml), progesterone (60 ng/ml), sodium selenite (40 ng/ml), thyroxine (40 ng/ml), triiodothyronine (30 ng/ml), putrescine (16 μg/ml) and insulin (5 μg/ml) (all from Sigma).

Embryonic spinal cord cell cultures

Sprague-Dawley rats from the University College London breeding colony were used throughout. The day of appearance of the vaginal plug was designated embryonic day zero (E0). Timed-mated females were killed by CO_2 asphyxiation and the embryos removed and killed by decapitation. The spinal cords were dissected away from surrounding tissue in Hepes-buffered minimal essential medium (MEM-H) and the meningeal membranes removed with watchmakers' forceps. The tissue was transferred to 2 ml Earle's balanced salt solution without calcium or magnesium (EBSS; Gibco-BRL) containing 0.0125% (w/v) trypsin (Boehringer Mannheim) and incubated at 37°C in 5% CO_2 for 30 minutes. The tissue was washed in DMEM containing 10% FCS (Gibco-BRL) to inhibit the trypsin, then transferred to fresh DMEM containing 10% FCS and 0.005% (w/v) DNase-I (Sigma). The tissue was immediately dissociated by gentle trituration with a Pasteur pipette. The resulting cell suspension was filtered through a 20 μm pore-diameter mesh and washed by centrifugation and re-suspension in DMEM containing 10% FCS. The number of live cells in a sample of the suspension was counted in a hemocytometer, using trypan blue (Sigma) exclusion as the criterion of viability. Cells were then plated on poly-D-lysine-coated 13 mm diameter glass coverslips in a 20 μl droplet. The cells were allowed to attach for 30 minutes at 37°C. 400 μl of BS medium was added, with or without 10 ng/ml PDGF-AA (Peprotech), and incubation continued at 37°C in 5% CO_2.

Immunoselection

Immunoselection was carried out by a modification of published procedures (Barres et al., 1992; Collarini, 1995). Three 60 mm diameter Petri dishes were incubated overnight at 4°C with 2 ml anti-Ig antibody solution - two with 10 μg/ml goat-anti-mouse IgG (Jackson Immunoresearch, Pennsylvania) and one with 10 μg/ml goat anti-rabbit IgG (Jackson Immunoresearch) in 50 mM Tris pH 9. The dishes were washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS) and incubated for several hours at room temperature with the appropriate antibodies for immunoselection (see below). The single-cell suspension of spinal cord cells (prepared as described above for spinal cord cultures) was washed by centrifugation and resuspension in MEM-H containing 0.5% FCS, passed over an uncoated Petri dish to remove macrophages, then sequentially over two dishes coated with a 1:10 dilution of RAN-2 hybridoma supernatant (Bartlett et al., 1981) to remove astrocytes and meningeal cells, then finally over a dish coated with a 1:200 dilution of anti-PDGFRα rabbit serum (Fretto et al., 1993). Each selection step was for 30 minutes at room temperature with occasional gentle swirling. The final dish was washed with MEM-H, then with EBSS, and the immunoselected PDGFRα$^+$ cells were removed with trypsin (0.125% [w/v] in EBSS), washed and resuspended in DMEM containing 10% FCS and plated at high density (1000 cells in a 3 μl droplet) on 6 mm diameter poly-D-lysine-coated glass coverslips in a 96-well tissue culture plate. The cells were allowed to settle for 30 minutes at 37°C before adding 35 μl BS-medium.

Antibody-mediated complement lysis

A suspension of E17 spinal cord cells was prepared as described above, except that FCS was omitted at all stages of the preparation to avoid non-specific serum-dependant complement lysis. The cell suspension was incubated simultaneously with rabbit complement (Cedar Lane Laboratories; diluted 1:12 in BS-medium) and A2B5 hybridoma supernatant diluted 1:5 in BS medium, at 37°C, 5% CO_2 for 45 minutes, with gentle inversion every 15 minutes. The cells were washed twice with DMEM and then once with DMEM containing 10% FCS. Approximately 40,000 viable cells (determined by trypan blue exclusion) were plated in a 20 μl droplet on a 13 mm diameter poly-D-lysine-coated glass coverslip. On the second day in vitro the complement treatment was repeated. The cells were incubated in antibody A2B5 (diluted 1:5 in BS medium) for 30 minutes at 37°C, then washed twice with DMEM. Rabbit complement (1:12 dilution in BS medium) was added for 30 minutes at 37°C, then the cells were washed twice with DMEM and incubated in fresh BS medium containing 0.5% FCS and 10 ng/ml PDGF-AA (Peprotech).

Immunocytochemistry

Cells on coverslips were lightly fixed in 2% (w/v) paraformaldehyde in PBS for 5 minutes at room temperature. The coverslips were washed three times in PBS, incubated in anti-PDGFRα rabbit serum (#3979) (Fretto et al., 1993; Nishiyama et al., 1996) diluted 1:100 in PBS for 30 minutes in a humid chamber at room temperature and rinsed three times in PBS. The cells were then incubated in goat anti-rabbit IgG (Jackson Immunoresearch) diluted 1:100 in PBS, for 30 minutes. Following this, the cells were incubated in one of the following primary antibodies: monoclonal antibody A2B5 (Eisenbarth et al., 1979), which labels a subset of gangliosides on the surface of O-2A progenitor cells (Raff et al., 1983); antibodies against the NG2 proteoglycan core protein (Stallcup and Beasley, 1987), which also label the surface of O-2A progenitors (Nishiyama et al., 1996), monoclonal antibody 04 (Sommer and Schachner, 1981), which labels sulphatide and other antigens on the surface of maturing O-2A progenitors (Bansal and Pfeiffer, 1992); monoclonal anti-galactocerebroside (GC; Ranscht et al., 1982), which reacts with an unidentified antigen in addition to GC (Bansal and Pfeiffer, 1992) on the surface of oligodendrocytes (Raff et al., 1978); monoclonal anti-glial fibrillary acidic protein (GFAP; clone GA-5, Sigma), an intermediate filament protein specific to astrocytes. Hybridoma cell supernatants were diluted 1:5 in PBS before use. The cells were post-fixed in 4% (w/v) paraformaldehyde in PBS and mounted under Citifluor (City University, UK). Labelled cells were viewed and photographed using a Zeiss Axioskop photomicroscope and Kodak T-Max 400 ASA film.

RESULTS

Characterization of PDGFRα[1]-immunoreactive cells from rat optic nerve and spinal cord

Oligodendrocyte progenitor cells were first identified and characterized in cultures of perinatal rat optic nerve cells (Raff et al., 1983; for reviews see Raff, 1989; Pfeiffer et al., 1994). They were named O-2A progenitors because they can differentiate into either oligodendrocytes or type-2 astrocytes, depending on the culture conditions; in defined medium containing at most 0.5% fetal calf serum (FCS) they give rise to oligodendrocytes, whereas in the presence of 10% FCS they give rise to type-2 astrocytes (Raff et al., 1983). These cell types can be distinguished in rat optic nerve cultures by mor-

phology and by reactivity with a range of antibodies. O-2A progenitors have a simple process-bearing morphology, often bipolar, and express gangliosides recognized by monoclonal antibody A2B5 (Eisenbarth et al., 1979; Raff et al., 1983). Oligodendrocytes are complex, multi-process-bearing cells that label with anti-galactocerebroside (GC) (Raff et al., 1978; Bansal and Pfeiffer, 1992). Type-2 astrocytes label with A2B5 and anti-glial fibrillary acidic protein (GFAP) (Raff et al., 1983). In the later stages of their differentiation into oligodendrocytes, O-2A progenitors assume a more complex shape and start to express surface antigens recognized by monoclonal antibody O4 (Sommer and Schachner, 1981, 1982; Bansal et al., 1992). Progenitors at this ($A2B5^+$, $O4^+$) stage have been termed pro-oligodendrocytes (Pfeiffer et al., 1994). The O4 antigen continues to be expressed by GC^+ oligodendrocytes. Both ($O4^-$, $A2B5^+$) and (O4

nantly ventral (Pringle and Richardson, 1993). Thus, the spatiotemporal distribution of PDGFRα+ cells closely matches that predicted for oligodendrocyte precursors by Warf et al. (1991).

We repeated the cell culture experiments of Warf et al. (1991), to test the prediction that development of oligodendrocytes in cultures of dorsal or ventral cells depends on the presence of PDGFRα+ precursor cells in the starting cell population. We bisected E14, E16 or E18 rat spinal cords longitudinally into dorsal and ventral halves, dissociated the cells and cultured them on glass coverslips in defined medium containing 0.5% FCS. Previous studies have shown that, under these conditions, cell cultures of embryonic rat brain (Abney et al., 1981), optic nerve (Raff et al., 1985) or spinal cord (Warf et al., 1991) first give rise to oligodendrocytes on or shortly before the equivalent of the day of birth (E21/P0), coinciding with the first appearance of significant numbers of oligodendrocytes in vivo (Abney et al., 1981; Miller et al., 1985; Jordan et al., 1989; Warf et al., 1991). Thus, cultures of E14 spinal cord cells generate substantial numbers of oligodendrocytes after 3-4 days (equivalent of E18), E16 cultures after 1-2 days and so on (Warf et al., 1991; our own unpublished data). Therefore, in the first series of experiments we fixed and labelled our spinal cord cultures after 16 hours with anti-PDGFRα and A2B5 to visualize presumptive oligodendrocyte progenitors, or with anti-GC on the equivalent of the day of birth to visualize oligodendrocytes. The results of these experiments are depicted in Fig. 2 and Table 1. No (PDGFRα+, A2B5+) process-bearing cells were present in E14 dorsal cultures after 16 hours, and no GC+ oligodendrocytes developed in parallel cultures during the next 7 days in vitro (Table 1; Fig. 2A,B,E,F). Small numbers of (PDGFRα+, A2B5+) process-bearing cells were present in E14 ventral cultures, and many oligodendrocytes developed within 7 days (Table 1; Fig. 2C,D,G,H). Significant numbers of (PDGFRα+, A2B5+) process-bearing cells were present in cultures of both ventral and dorsal cells from E16 and E18 spinal cords after 16 hours in vitro, and many oligodendrocytes developed in these cultures by the equivalent of the day of birth (Table 1). These data confirm the findings of Warf et al. (1991) and in addition show that the presence of (PDGFRα+, A2B5+) process-bearing cells in the starting cell population correlates with the ability of the cultures to give rise to oligodendrocytes in the longer term. In all these spinal cord cultures, as in optic nerve cultures, there were small numbers of (PDGFRα+, A2B5−) flat meningeal cells, which

Table 1. PDGFRα[1] progenitors and GC[1] progenitors in cultures of rat spinal cord cells

	PDGFRα+ cells per coverslip after 16 hours in vitro		GC+ oligodendrocytes per coverslip at equivalent of P0	
Age	Dorsal	Ventral	Dorsal	Ventral
E14	0	44±9	0	1387±245
E16	46±3	212±18	368±71	1908±204
E18	1360±90	1865±516	262±170	323±55

E14 rat spinal cords were dissected into dorsal and ventral halves and cultured separately on glass coverslips as described in Materials and methods. After 16 hours the cells were fixed and labelled with anti-PDGFRα to visualize presumptive oligodendrocyte progenitors. Parallel coverslips were cultured longer, until the equivalent of the day of birth (i.e. E14 + 7DIV, E16 + 5DIV, E18 + 3DIV; DIV means days in vitro), then fixed and labelled with anti-GC to visualize oligodendrocytes. PDGFRα+ cells were present initially, and oligodendrocytes developed subsequently, in all cultures except cultures of E14 dorsal cells. Tabulated are mean numbers of cells and standard deviations of three independent experiments conducted in duplicate or triplicate. There was not a strict correlation between the number of PDGFRα+ cells in the starting population and the number of oligodendrocytes that developed in culture. For example, fewer oligodendrocytes developed in the E18 cultures than in the E16 cultures, despite the fact that there were more PDGFRα+ cells initially present in the E18 cultures. Part of the reason for this is presumably that the E18 cells were cultured for a shorter time than the E16 cells (3 days rather than 5 days in vitro). However, our impression was that there was more cell damage and death caused during dissociation of E18 than E16 spinal cords, probably reflecting increased mechanical damage to neurons. Reduction in the number of neurons in the cultures might have affected the rate at which oligodendrocytes differentiated or or the proportion that survived in the cultures.

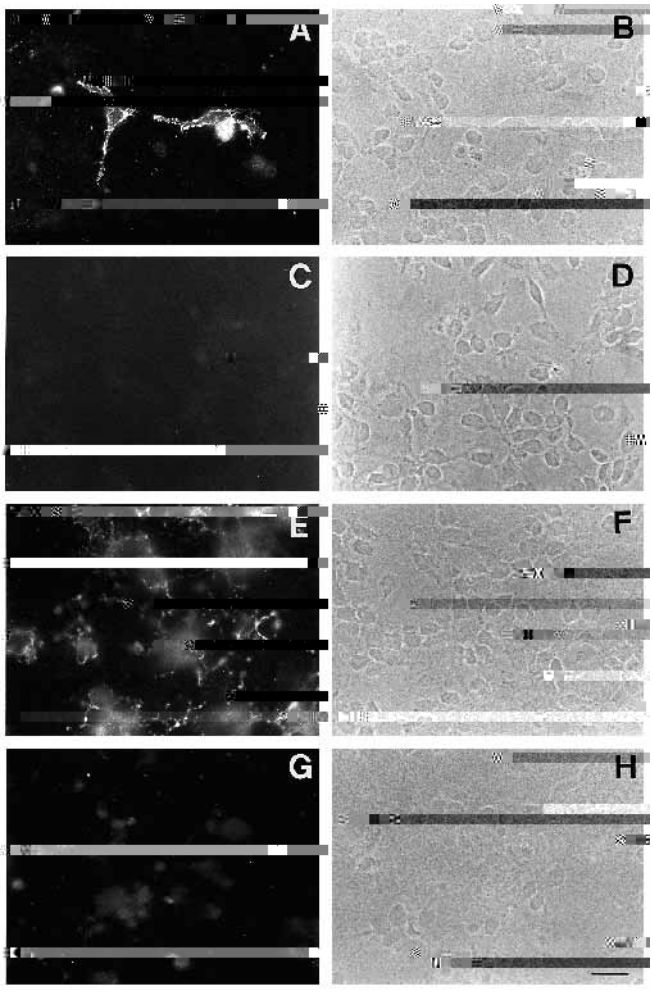

Fig. 2. PDGFRα+ precursors and oligodendrocytes in cultures of E14 rat ventral and dorsal spinal cord cells. E14 cords were divided longitudinally into ventral and dorsal halves, dissociated and cultured on glass coverslips in BS medium containing 0.5% FCS with or without 10 ng/ml PDGF-AA. Cultures were immunolabelled with anti-PDGFRα (A-D) or anti-GC (E-H) and photographed under fluorescence or phase contrast optics. After 16 hours in vitro, ventral cultures contained small numbers of PDGFRα+ cells (A,B) but dorsal cultures did not (C,D). After 7 days in cultures, many GC+ oligodendrocytes had developed in ventral cultures (E,F) but few or none developed in dorsal cultures (G,H). See Table 1 and Fig. 3 for further information. Scale bar, 50 μm.

we omitted from the analysis. In both dorsal and ventral E14 cultures, unlike optic nerve cultures, there were also many ($A2B5^+$, $PDGFR\alpha^-$) process-bearing cells after 16 hours in vitro (not shown). These presumably represent neural precursors of some sort; A2B5 is known to label many immature neurons, for example (Eisenbarth et al., 1979). Lower numbers of these cells were still present in E16 cultures, but they were very rare at E17 or later (see below), possibly because they differentiate and lose A2B5 immunoreactivity between E14 and E17.

The simplest explanation for these results is that oligodendrocyte progenitors, possibly the ($PDGFR\alpha^+$, $A2B5^+$) process-bearing cells described, arise first in the ventral half of the E14 spinal cord and migrate into the dorsal half by E16. However, we were concerned that oligodendrocyte progenitor cells might be missed in the E14 dorsal cultures if, for example, they were very infrequent in the E14 dorsal cord or if they were less able to survive in the dorsal cultures than the ventral cultures. We therefore repeated the E14 spinal cord cultures, plating the cells at a much higher density (75,000 instead of 5,000 cells per coverslip) and scoring the presence of ($PDGFR\alpha^+$, $A2B5^+$) progenitor cells and GC^+ oligodendrocytes at more frequent intervals. We also cultured the cells for longer – until the equivalent of P5 or P7 – in case oligodendrocytes develop somewhat later in dorsal cultures than in ventral cultures. The outcome of these experiments was qualitatively the same as before. E14 ventral cultures contained a small number of $PDGFR\alpha^+$ cells after overnight incubation, and these multiplied dramatically over the 12-day culture period (Fig. 3). Oligodendrocytes first appeared in these cultures 7 days after plating and increased in number thereafter. E14 dorsal cultures, by contrast, contained few or no $PDGFR\alpha^+$ cells after overnight incubation, as expected, and usually no $PDGFR\alpha^+$ cells were present in these cultures after the first week of culture (Fig. 3). In some cultures small numbers of $PDGFR\alpha^+$ cells were visible after 1 week, and these usually occurred in isolated clusters, as if they had arisen by clonal expansion of one or a few cells in the starting cultures. We rarely found oligodendrocytes in the dorsal E14 cultures, even after 2 weeks in culture. Invariably, if no $PDGFR\alpha^+$ cells were detected at the outset of a particular experiment (i.e. after 16 hours in culture), then no oligodendrocytes developed subsequently in the parallel cultures. The outcome of all these experiments was qualitatively the same whether PDGF-AA was added to the medium or not, although more $PDGFR\alpha^+$ cells and oligodendrocytes developed in the presence of PDGF. In separate experiments we added basic fibroblast growth factor (10 ng/ml) or Sonic hedgehog (7×10^{-9} M) to the cultures, without changing the results.

Therefore, we have confirmed the central finding of Warf et al. (1991), that the ability of spinal cord cells to generate oligodendrocytes starts in the ventral cord at E14 and later progresses to the dorsal cord. Moreover, the presence or absence of PDGFRα-immunoreactive cells in spinal cord cultures correlates with the distribution of PDGFRα mRNA-positive cells visualized by in situ hybridization, and predicts the ability of the cultures to generate oligodendrocytes in vitro. The data is consistent with our hypothesis that $PDGFR\alpha^+$ cells are the progenitors of oligodendrocytes, and that these cells begin life in the ventral region of the spinal cord and subsequently proliferate and migrate dorsally.

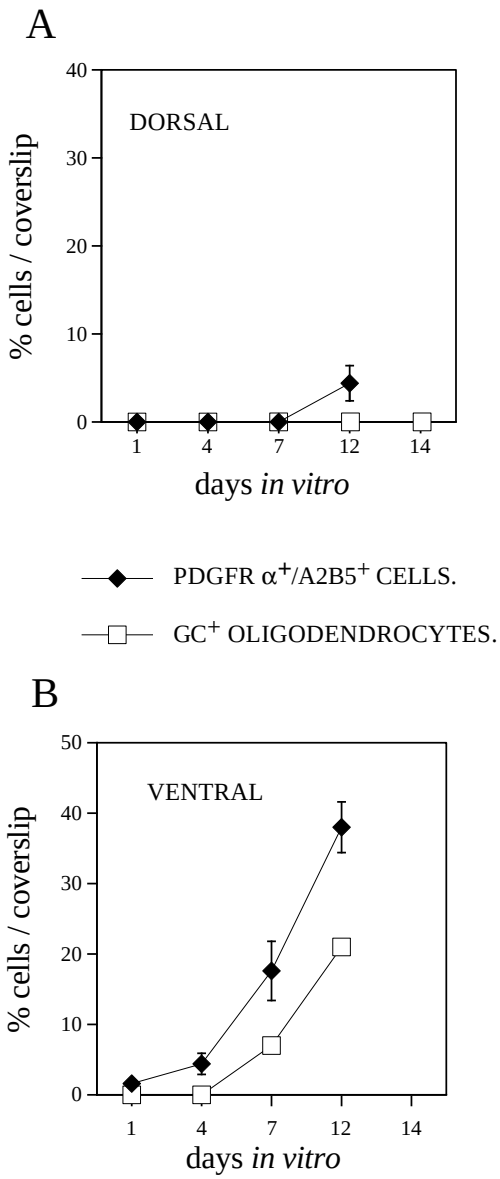

Fig. 3. Time course of appearance of $PDGFR\alpha^+$ precursors and GC^+ oligodendrocytes in cultures of E14 dorsal or ventral spinal cord cultures. E14 spinal cords were divided into dorsal and ventral halves, dissociated and plated at high density on glass coverslips (75,000 cells per coverslip) in BS medium containing 0.5% FCS. After various culture periods, the cells were fixed and immunolabelled with anti-PDGFRα together with A2B5, or anti-GC antibodies. The cells were given a final wash in Hoechst 33258 (Sigma) to label all cell nuclei. The numbers of ($PDGFR\alpha^+$, $A2B5^+$) process-bearing cells and GC^+ oligodendrocytes were counted (triplicate coverslips from two independent experiments) and expressed as a percentage of the total number of cells. Small numbers of $PDGFR\alpha^+$ cells were present in ventral cultures at early

All PDGFRα[1] process-bearing cells in embryonic rat spinal cord are O-2A progenitors

In situ hybridization studies show that there are increasing numbers of scattered cells that express PDGFRα mRNA in the rat spinal cord after E14 (Pringle and Richardson, 1993). We had previously suggested that these cells might be oligodendrocyte progenitors and the culture experiments described above are consistent with this. To test this idea directly, we immunoselected PDGFRα$^+$ cells from freshly prepared cell

approximately 30-fold less than in any of the controls (Table 2; Fig. 5A-D). The depleted cell populations were maintained in defined, low-serum medium with or without PDGF-AA for a further 4 days, until the equivalent of the day of birth, to allow time for oligodendrocytes to develop in vitro. At the end of the culture period, the cells were fixed and stained with anti-GC to visualize oligodendrocytes. The cultures depleted of ($A2B5^+$, $PDGFR\alpha^+$) process-bearing cells contained approximately 25-fold less GC^+ oligodendrocytes than either of the control cultures (Table 2; Fig. 5E-H). Therefore, the reduction in the number of oligodendrocytes approximately matched the reduction in the number of ($A2B5^+$, $PDGFR\alpha^+$) precursors in the starting population.

DISCUSSION

The purpose of the experiments described in this paper was to test the idea that $PDGFR\alpha^+$ cells in the embryonic rat spinal cord are oligodendrocyte progenitors, and to assess the contribution these $PDGFR\alpha^+$ cells make to oligodendrogenesis in the cord. We found that essentially all $PDGFR\alpha^+$ process-bearing cells in perinatal rat optic nerve or late embryonic spinal cord cultures co-labelled with antibody A2B5, an established marker of oligodendrocyte progenitors in optic nerve cultures. Conversely, nearly all $A2B5^+$ cells in cultures of optic nerve or spinal cord (after E17) co-labelled with anti-PDGFRα. $PDGFR\alpha^+$ cells, immunoselected from suspensions of dissociated E17 spinal cord cells with an antibody against

(1991), who reported that the ability of rat spinal cord cells to generate GC^+ oligodendrocytes in culture was acquired first by

vivo. If so, and if most or all spinal cord oligodendrocytes develop from PDGFRα$^+$ precursors as our present study suggests, one would predict that PDGF or PDGFRα null mutants might be severely affected in oligodendrogenesis in the cord. In keeping with this prediction, we have found that there are only about 5% of the normal number of PDGFRα$^+$ progenitor cells in the in the spinal cords of PDGF-A knockout mice and a greatly reduced number of oligodendrocytes (Calver, A., Hall, A., Fruttiger, M., Yu, W.-P., Boström, H., Willetts, K., Heath, J. K., Betsholtz, C. and Richardson, W. D., unpublished data). This provides further indirect evidence that oligodendrogenesis in the spinal cord relies heavily on the PDGFRα$^+$ lineage that has been the subject of this investigation.

We thank our friends and colleagues in the LMCB for help, encouragement and advice, especially Ellen Collarini for help with the immunopanning procedure. We acknowledge a gift of anti-NG2 antibodies from Bill Stallcup. This work was supported by the UK Medical Research Council and the Multiple Sclerosis Society of Great Britain and Northern Ireland.

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