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Proliferation and differentiation potential of rat forebrain oligodendroglial progenitors both *in vitro* and *in vivo*

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Summary

We have followed the development of the O-2A progenitor cell from the neonatal rat forebrain, both in dissociated cell culture and in cryostat sections, using immunocytochemical techniques employing a panel of antibodies that recognise the cells at different stages of their development. This included the monoclonal antibody LB_1, which binds to the surface ganglioside G_{D3} expressed on O-2A progenitor cells. In secondary cultures enriched for O-2A progenitors maintained in a serum-free chemically defined medium, a large proportion of the cells are primed to differentiate into oligodendroglia and go on to express the oligodendroglial specific surface glycolipid galactocerebroside (GC) and then the myelin proteins CNP and MBP. However, a significant proportion of immature bipolar G_{D3}^+ cells remained after 6 days in secondary culture. It appears that not all the O-2A progenitors in our cultures differentiate immediately and some cells remain in an undifferentiated state and divide to replenish progenitor numbers. We have also identified in our cultures a small apolar G_{D3}^- cell, which when isolated differentiated into a G_{D3}^+ bipolar O-2A progenitor cell. We have termed this cell type a preprogenitor. The differentiation of this cell type into O-2A progenitors may be the source of the immature G_{D3}^+ cells present at the later stages of our secondary cultures. The proliferative profile of the cultures was studied using 5'bromo-2-deoxyuridine (BrdU) incorporation as an index of mitosis. Only the immature, bipolar O-2A progenitors were seen to divide at any time in serum-free culture. Neither the more mature multipolar O-2A cells nor the oligodendroglia were seen to divide. The developmental profile of the O-2A cells in the rat forebrain *in vivo* showed a largely similar progression to that in culture, with a time lag of at least 6 days between G_{D3} expression and the onset of myelination. BrdU incorporation studies *in vivo* also showed that the G_{D3}^+ progenitor cell is mitotic whereas the GC^+-expressing oligodendroglia is not. We have shown that there are several significant alterations in the timing of antigen expression in both O-2A progenitors and oligodendroglia *in vitro* compared to that seen *in vivo*.

Key words: oligodendroglia, astroglia, progenitors, myelination, development, cell culture.

Introduction

To increase our understanding of the process of myelination and the repair of demyelinated lesions of the central nervous system (CNS), such as those seen in Multiple Sclerosis (MS), the various stages of the differentiation of oligodendroglia and their precursors, both *in vivo* and *in vitro*, need to be characterised and their potential for repair and regeneration investigated. The early development of a bipotential glial progenitor cell has been extensively characterised in dissociated cell cultures derived from neonatal rat optic nerve (Raff, 1989). These cells have been shown to differentiate into oligodendroglia when grown in culture medium without foetal calf serum (FCS) but to become process-bearing astroglia (type-2 astroglia) when grown in culture medium containing 10–20% FCS (Raff *et al.* 1983). This progenitor cell has thus become known as the O-2A progenitor. Similar findings have been reported for glial progenitors grown in cultures derived from cerebral hemispheres (Behar *et al.* 1988) and cerebellum (Levi *et al.* 1987), although cells from these regions have not been so extensively characterised.

The study of the differentiation of glial progenitors into oligodendroglia has largely depended on the use of sequential antigenic markers to follow the development of the cells in dissociated cell culture. The O-2A progenitor cell has been defined as a bipolar cell that expresses one or more of the gangliosides recognised by the antibody A2B5 (Raff *et al.* 1983; Eisenbarth *et al.* 1979; Dubois *et al.* 1990) on its surface, or the single ganglioside G_{D3} (Levi *et al.* 1987), but does not yet express the myelin lipid galactocerebroside (GC). These cells then pass through a transition stage during

which the gangliosides are coexpressed with GC. Gangliosides recognised by A2B5 and G_{D3} ganglioside are lost from the surface of the cell as it differentiates into an oligodendroglia. O-2A progenitors have also been shown to express the intermediate filament protein vimentin during the early stages of differentiation (Behar *et al.* 1988). When these progenitors are grown in serum-containing medium, they begin to express glial fibrillary acidic protein (GFAP) and assume the stellate morphology of type-2 like astroglia, while retaining vimentin intermediate filaments (Raff *et al.* 1983; Levi *et al.* 1987).

Although the differentiation of the O-2A progenitor cell into oligodendroglia and type-2 astroglia has been well characterised in the optic nerve *in vitro*, its relevance to development *in vivo* is not well established. In a recent study we followed the differentiation pattern of the G_{D3}-expressing glial progenitor in the developing rat cerebellum *in vivo* using sequential antigenic markers to follow the cells through to the mature myelin-producing oligodendroglia (Reynolds and Wilkin, 1988). The G_{D3}-expressing glial progenitors in the developing rat cerebellum arise close to the subventricular zone of the 4th ventricle and the temporal changes in their position suggest that they migrate to their final position before further differentiation. They express both G_{D3} and GC transitionally before growing multiple processes and expressing 2,3-cyclic nucleotide-3-phosphohydrolase (CNP) and finally myelin basic protein (MBP), shortly before the onset of myelin formation. Our results using *in vivo* ^{3}[H]thymidine incorporation suggest that, of the various stages of differentiation through which the cells pass, only the G_{D3}-expressing progenitor is substantially mitotic during normal development in the cerebellum (Reynolds and Wilkin, 1991). Both our study in the cerebellum (Reynolds and Wilkin, 1988) and a similar study of several other regions (Levine and Goldman, 1988*b*) failed to observe a coincidence of G_{D3} and GFAP immunolabelling at any stage, the antigenic phenotype used to identify type-2 astroglia in culture.

In this study, we describe the establishment of a highly enriched G_{D3}-expressing glial progenitor culture from rat forebrain and the development of these cells with respect to their expression of antigens reflecting increasing maturity and also their mitogenic profile. Comparisons with the *in vivo* development of cells of this lineage are made with a comprehensive study of oligodendroglial phenotype progression in cryostat sections of rat forebrain.

Materials and methods

Cell culture

Dulbecco's Modified Eagle's Medium (DMEM) with 10% FCS (Flow Laboratories), penicillin (200 i.u. ml^{-1}) and streptomycin (200 $\mu g\,ml^{-1}$) was used unless otherwise stated. Primary mixed glial cultures were made under aseptic conditions as follows. Neonatal Sprague Dawley rat pups (less than 12 h old) were decapitated and the forebrains removed and placed in culture medium in a Petri dish. After removal of the hypothalamus, thalamus and striatum, cerebral cortices were transferred to 5–10 ml of culture medium in a second dish and chopped coarsely with a razor blade. The pieces were then mechanically dissociated by trituration in a 10 ml plastic pipette (Falcon). The resulting cell suspension was centrifuged (1000 revs min^{-1}, 5 min) and the pellet resuspended in culture medium. The cell density was adjusted to 2.5×10^5 ml^{-1} and cells were plated at 5×10^4 cm^{-2} in 150 cm^2 tissue culture flasks (Flow Laboratories), which had been previously coated with poly-L-lysine. Cultures were maintained at 37°C in a humid atmosphere containing 5% CO_2 and the culture medium changed every 3–4 days.

After 12 days in culture, O-2A progenitor cells and oligodendroglia growing on top of the astrocyte monolayer were removed by a modification of the technique described by McCarthy and DeVellis (1980). Flasks were shaken on an orbital shaker (2.5 cm throw) for 30 min at 150 revs min^{-1} at 37°C to remove any dead or loosely adherent cells. The medium was changed to DMEM with 15% FCS to enhance cell survival during the shaking procedure, and flasks were left to equilibrate in the incubator for 60 min. They were then shaken at 250 revs min^{-1} at 37°C for 17–18 h. Medium was decanted and filtered through 70 μm and then 25 μm nylon mesh. The resulting cell suspension was centrifuged (1000 revs min^{-1}, 5 min) and the pellet resuspended in culture medium, transferred to an untreated 150 cm^2 tissue culture flask and incubated for 30 min at 37°C. During this time, microglia, which constituted about 40% of the cells, but not progenitor cells or oligodendroglia, adhered to the plastic. Oligodendroglia were removed by treatment with a monoclonal antibody to galactocerebroside (GC; Ranscht *et al.* 1982) and complement as follows. Floating cells were recovered by centrifugation and resuspended in 5 ml Earle's Balanced salts (EBS) containing 0.3% bovine serum albumin (BSA), 0.004% DNAase I, 10% normal rabbit serum as a source of complement and 20 μl anti-GC (IgG fraction). They were then incubated in a shaking water bath at 37°C for 60 min, washed in culture medium, spun down, resuspended and counted in a haemocytometer. Cells were plated at a density of 2.5×10^4 cm^{-2} on poly-L-lysine-coated glass coverslips in 24-well tissue culture plates in DMEM with 10% FCS. About 10^5 cells from the suspension were used to confirm the absence of oligodendroglia by GC immunohistochemistry (see below). After 24 h the medium was changed to a serum-free medium consisting of a DMEM/Ham's F12 mixture (1:1), insulin (10 $\mu g\,ml^{-1}$), transferrin (50 $\mu g\,ml^{-1}$), triiodothyronine (15 nM), sodium selenite (30 nM), penicillin (200 i.u. ml^{-1}) and streptomycin (200 $\mu g\,ml^{-1}$). Medium was not changed thereafter. In some experiments, medium remained DMEM with 10% FCS throughout secondary culture, fresh medium being added every 3–4 days.

In several experiments, the isolated cells were treated with antibodies to both G_{D3} and GC with complement to remove both oligodendroglia and O-2A progenitors from the cell suspension and then plated at 2.5×10^4 cm^{-2} as above.

Antibodies

Mouse monoclonal antibody (mAb) LB_1, which binds to G_{D3} ganglioside, has been described and characterised by us previously (Curtis *et al.* 1988); mAb anti-galactocerebroside (anti-GC) was provided by Dr B. Ranscht (San Diego). Rabbit polyclonal antibodies to MBP have been produced and characterised by us previously (Reynolds *et al.* 1989). Rabbit polyclonal antibodies to CNP were provided by Dr F. A. McMorris (Philadelphia; Raible and McMorris, 1989). Mouse mAb OX42 was purchased from Serotec. Mouse mAb

anti-5′-bromo-2-deoxyuridine (anti-BrdU), rabbit polyclonal antibody to GFAP and mouse mAb to vimentin were purchased from DAKOPATTS (Denmark). Fluorochrome-conjugated second antibodies were purchased from Cappel Labs, Nordic and Serotec.

Immunostaining of cell cultures

Cell cultures were labeled using indirect double immunofluorescence. For staining with antibodies to cell surface components (G_{D3}, GC and OX42), live cells growing on coverslips were incubated with primary antibody diluted in Earle's balanced salts (EBS) with 0.3 % BSA and 5 % normal goat serum (NGS) for 20 min, followed by fluorochrome-conjugated goat anti-mouse IgM, IgG_2 or IgG_3 antibodies for 15 min. For double immunofluorescence using two surface antigens, the cultures were then incubated as above with the second primary antibody for 20 min followed by the appropriate fluorochrome-conjugated anti-mouse immunoglobulin. Cells were fixed in 4 % paraformaldehyde in PBS for 20 min. For staining with antibodies to membrane bound or intracellular antigens (CNP, MBP, GFAP and Vimentin), cells were permeabilised after fixation by incubation with 0.25 % Triton X-100 in PBS (5 min). Primary antibodies were added for 45 min, followed by the appropriate fluorochrome-conjugated goat anti-mouse, goat anti-rabbit or swine anti-rabbit immunoglobulin antibodies for 30 min. Coverslips were rinsed once in PBS and mounted in glycerol/PBS (9:1) with 2.5 % diazabicyclo-octane to reduce fading of the immunofluorescence. Each step was followed by three washes in EBS with 0.3 % BSA. All primary antibodies were diluted in EBS with 0.3 % BSA and 5 % NGS at the following dilutions: mAb G_{D3} ascites (LB1; IgM) 1/500; mAb GC supernatant (IgG_3) 1/10; mAb vimentin supernatant (IgG_1) 1/10; CNP, MBP and GFAP affinity-purified polyclonal antibodies 1/200; mAb OX42 (IgG_2) 1/100.

Immunostaining of cell suspensions was carried out in EBS with 0.3 % BSA, 5 % NGS and 0.004 % DNAase I. Aliquots of 10^5 cells were suspended in 200 μl of primary antibody diluted as above and incubated for 20 min. Cells were then spun down, resuspended and pelleted in wash buffer, and then incubated in 200 μl of appropriate second antibody (15 min). This was repeated with primary antibody to a second surface antigen if required. Fixation was carried out in suspension in 4 % paraformaldehyde in PBS as above. Cells were allowed to adhere to poly-L-lysine-coated coverslips. For immunostaining using intracellular antigens, cells were treated with 0.25 % Triton X-100 in PBS for 5 min and incubated with primary antibody and conjugate as above. All coverslips were mounted as above.

Bromodeoxyuridine incorporation in vitro

BrdU (10 μM) was added to culture medium for 18 h after which coverslips were washed thoroughly in EBS, immunostained for the appropriate antigen as described above and fixed and permeabilised with Triton X-100 as before. Cells were then treated with 2 N HCl for 10 min to denature the DNA (the anti-BrdU antibody used only recognises single-stranded DNA), followed by 0.1 M sodium borate pH 8.5 for 10 min. The primary anti-BrdU antibody (diluted 1:10) was added for 45 min, followed by the fluorochrome-conjugated goat anti-mouse IgG_1 for 30 min.

Cryostat sections

Sprague Dawley rats of the following ages were studied: embryos of 19–20 days gestation (designated E19-E20), newborn pups (designated P0) and pups aged P1, P2, P3, P4, P6, P7, P8, P9, P10, P12, P15, P17, P20 and P21. The animals were anaesthetised with sodium pentabarbitone and perfused, through the left ventricle, with phosphate-buffered saline (PBS) (5–10 ml) at 37 °C and then with 4 % paraformaldehyde in PBS (5–20 ml) also at 37 °C. Rats were decapitated and their forebrains removed. A thick coronal section was removed from the middle of the forebrain and postfixed in 4 % paraformaldehyde in PBS for up to 30 h at 4 °C. Blocks were cryoprotected in 30 % sucrose in PBS overnight at 4 °C. 5 μm cryostat sections were cut and allowed to adhere to glass slides precoated with gelatin/chrome alum for at least an hour in a humid box before immunostaining.

Immunostaining of cryostat sections

Sections were incubated in sodium borohydride (0.5 mg ml^{-1}; 2×5 min) to reduce non-specific staining, washed thoroughly and incubated with primary antibody diluted in PBS overnight in a humid chamber. This was followed by a 60 min incubation with fluorochrome-conjugated second antibodies. Sections to be double labelled were incubated with the appropriate primary antibody for 3 h followed by fluorochrome-conjugated second antibody for 1 h and slides were then mounted as above. Treatments preceding the incubation with primary antibody were as follows: LB1 and GC, no treatment; CNP and MBP 10 min with methanol at 4 °C; GFAP and vimentin, 10 min with 0.25 % Triton X-100 in PBS. Each step was followed by two washes in PBS. All antibody dilutions were as for immunostaining of cultured cells.

Bromodeoxyuridine incorporation in vivo

Sprague Dawley rats were given two intraperitoneal injections, 90 min apart, of 100 μg g^{-1} body weight BrdU in saline. Tissue was prepared as above 90 min after the second injection and cryostat sections cut as above. In some experiments, rats were given four injections over a 6 h period. Sections to be stained for BrdU were treated as above, incubated with either LB1 or GC antibodies and treated with 4 N HCl for 15 min, followed by two 5 min washes in 0.1 M sodium borate, pH 8.5, and 1 wash in PBS, before incubation with anti-BrdU antibody (3 h) and GAMIgG_1 rhodamine-conjugated second antibody (1 h).

Results

Development of progenitors and oligodendroglia in SFM culture

An enriched population of G_{D3} expressing O-2A progenitor cells was obtained from mixed glial cultures by a modification of the method of McCarthy and DeVellis (1980). Microglia and any flat cells were successfully removed by selective adhesion onto untreated plastic dishes. GC^+ oligodendroglia were removed by treatment with anti-GC antibody and complement. Cells from the resulting population were immunostained in suspension. The majority of cells (76 %) were $G_{D3}{}^+$ with only 3 % GC cells (Fig. 1). Only around one third of the progenitor cells were seen to express vimentin. This may be due to the difficulty of localising intermediate filaments to a cell with no processes. $Vimentin^+$ cells amounted to 36 % of the total, half of which were the $G_{D3}{}^+$ O-2A progenitor cells. Contaminating $GFAP^+$ astrocytes appeared to be mostly type-2-like in that they also expressed G_{D3} (7 % of total cells). Only 2 % of cells were $GFAP^+/G_{D3}{}^-$ and were presumably type-1-like, which are also

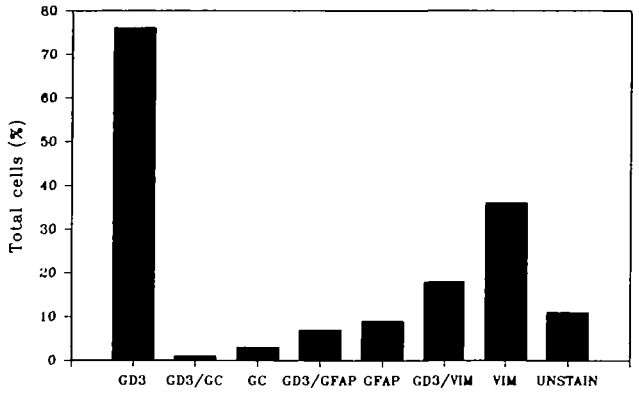


Fig. 1. Antigenic phenotype of cells isolated from 12 day rat forebrain mixed glial cultures by a shaking method modified from that of McCarthy and deVellis (1980). Cells were stained in suspension immediately following isolation. Numbers are percentages of total cells. At least 400 cells were counted per antigen per 13 mm coverslip in each of 2–6 separate experiments. Errors were less than 10 % in all cases.

vimentin$^+$. There remained 11 % of cells that were vimentin$^+$ only, together with 9 % of cells that were unstained with any of the above markers. It is likely that a small number of these were microglia, but we were unable to stain these cells successfully with OX42 in suspension. The discrepancy between total labelled cells and total cells will be addressed in a later section.

Fig. 2 shows the antigen profile of the secondary cultures with time. After 24 h in secondary culture (designated day 1), G_{D3}^+ cells had assumed the bipolar morphology characteristic of O-2A progenitor cells (Fig. 3A,B and C). In a separate study, these cells were shown to express GAP-43, which was lost as the cells acquired GC (Curtis, Hardy and Reynolds, unpublished data). Few cells expressed a more differentiated appearance. Only 2 % of the total cell population were GC$^+$ oligodendroglia at this stage. These were small cells with many short processes, half of which were G_{D3}^+/GC$^+$, indicating an immature population of cells recently differentiated from O-2A progenitors. Contaminating cell types were astrocytes (5 %) and OX42$^+$ microglia (3 %). The percentage of astrocytes and OX42$^+$ cells in the cultures remained below 5 % throughout the time studied.

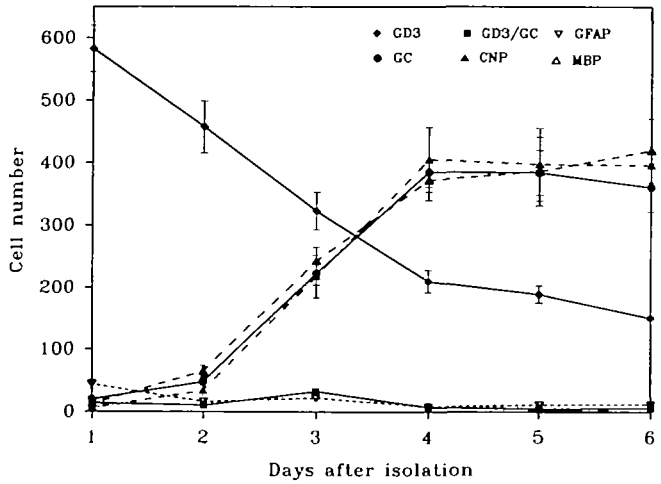


Fig. 2. Antigenic profile of O-2A progenitor-enriched secondary cultures in serum-free medium. Cells were immunostained on each day of secondary culture. 20 fields (40×) were counted per coverslip. Numbers are means±S.E.M. of 5–24 coverslips from 2–5 separate experiments.

By day 2, 24 h after the cultures had been switched to SFM, progenitors had started to show a more differentiated morphology. They became largely multipolar, some with an interconnecting network of branches characteristic of oligodendroglia (Fig. 3D). Less than 2 % of these cells co-expressed GC; oligodendrocytes only accounted for 7 % of total cells at this stage.

On day 3, the number of GC$^+$ cells had risen dramatically, now accounting for 38 % of total cells. These had the characteristic multiprocess-bearing morphology of oligodendroglia in culture (Fig. 3F). G_{D3}^+ progenitor cells now fell into two categories: most G_{D3}^+ cells were highly branched with multiple processes, while others were small and either apolar or bipolar in appearance, resembling immature progenitor cells. The number of G_{D3}^+/GC$^+$ cells rose proportionately reaching its peak at this stage. Many of the cells at this stage displayed an elaborate, multipolar morphology (Fig. 3E,F). The myelin proteins CNP and MBP were also expressed by all GC$^+$ oligodendroglia at this stage. CNP was present in the cytoplasm of the oligodendroglia in both the cell body and the processes whereas MBP was first seen in the cell body only. CNP$^+$/GC$^-$ cells were seen as well as GC$^+$/CNP$^-$ cells but the majority were both GC$^+$ and CNP$^+$.

By day 4 the number of GC$^+$ oligodendroglia had now reached 53 %. CNP and MBP expression continued to follow that of GC; almost all GC$^+$ cells were also CNP$^+$ (Fig. 4A,B). The proportion of immature bipolar progenitor cells had increased and these could be seen amongst mature oligodendroglia (Fig. 4C,D). Oligodendroglia showed intricate networks of processes (Fig. 4E). The intensity of both CNP and MBP expression had increased and MBP could now be seen in oligodendroglial processes as well as in the cell body (Fig. 4F). More mature cells could be seen producing characteristic myelin-like membrane although this was not particularly common in our cultures. The number of oligodendroglia in the cultures over the next two days remained relatively constant as did the proportion of G_{D3}^+ progenitor cells.

BrdU incorporation in vitro

Proliferating cell types in the cultures were studied using the thymidine analogue 5′-bromo-2-deoxyuridine (BrdU). Cultures were incubated with BrdU (10 μM) for 18 h on days 0 and 4 of secondary culture. Cells were dual stained for either G_{D3}/BrdU or GC/BrdU. At day 0/1, when cultures are growing in serum-containing medium, 32 % of cells expressing G_{D3}

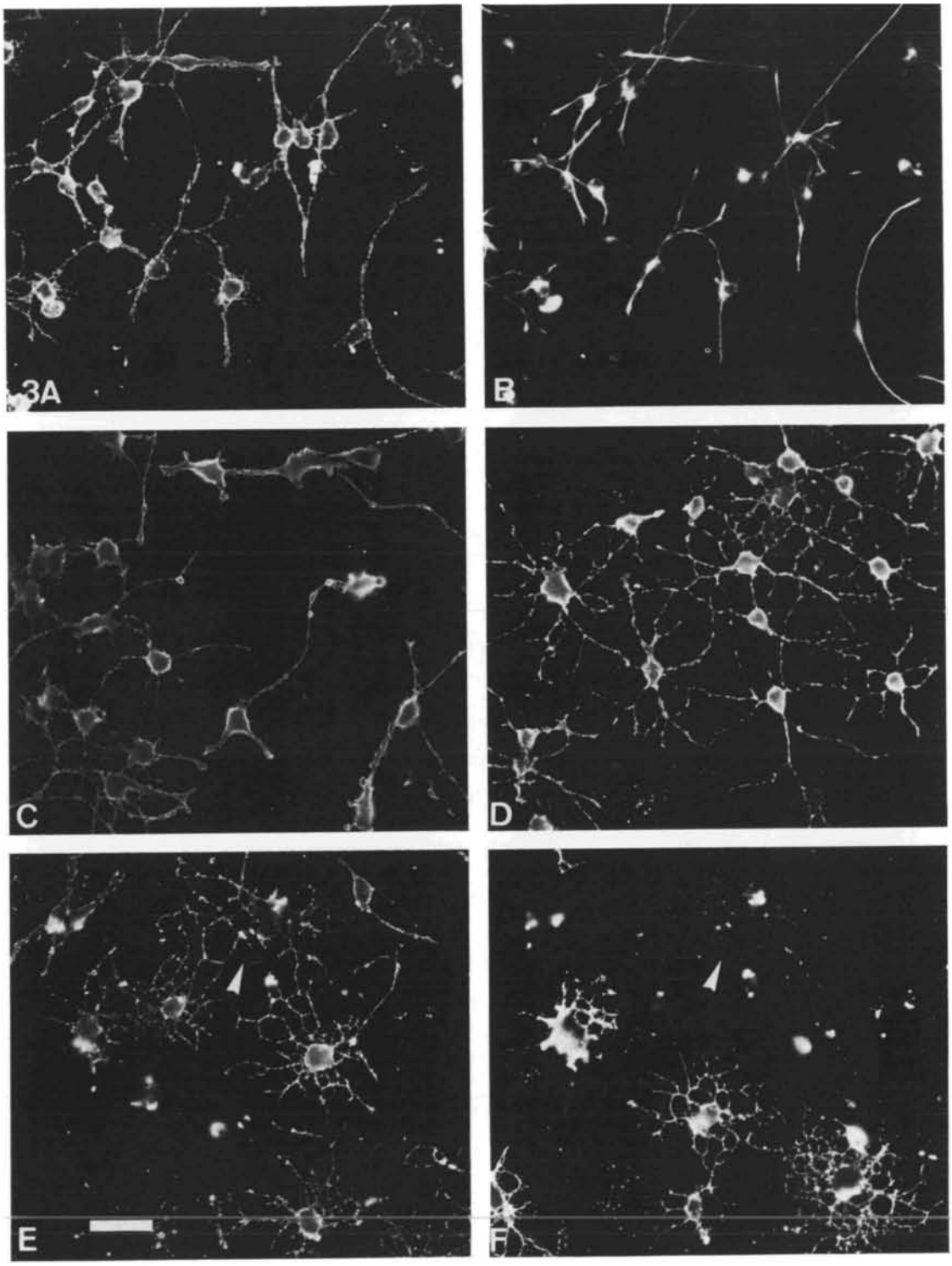


Fig. 3. Double-immunofluorescent labelling of progenitor-enriched secondary cultures on day 1 (A,B,C), day 2 (D) and day 3 (E,F) with antibodies to G_{D3} and vimentin (A,B); G_{D3} and GC (E,F) and single labelling with antibodies to G_{D3} (C and D). At 1 day after secondary culture progenitors are largely bipolar and express both G_{D3} and vimentin (A,B). The G_{D3} immunostaining is clearly localised on the surface of the cells (C). By day 2, progenitors assume a more multipolar appearance (D). By day 3, many have ceased to express G_{D3} but have acquired GC (E,F). Some cells express both antigens (arrowheads). Bipolar G_{D3}^+/GC^- cells can still be seen. Scale bar=40 μm.

had incorporated BrdU (Fig. 5). These were cells of bipolar morphology, the multipolar G_{D3}^+ cells had failed to incorporate BrdU (Fig. 6A). At this stage, no GC^+ cells could be seen co-labelled with anti-BrdU. On day 4/5, the numbers of G_{D3}^+ cells had decreased but now 62% of these had incorporated BrdU during the

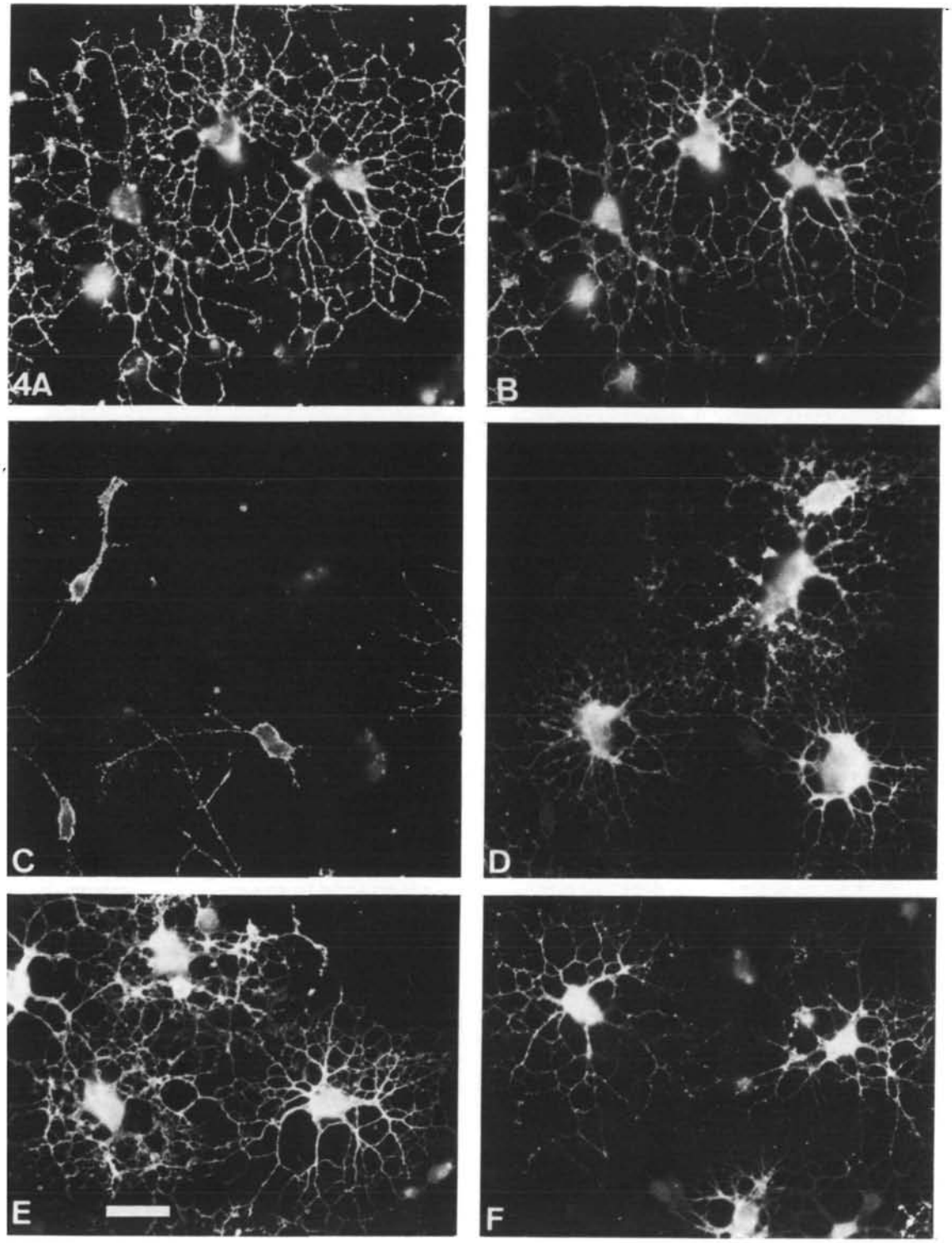


Fig. 4. Double-immunofluorescent labelling of progenitor-enriched secondary cultures on day 4 (A,B) and day 6 (C,D) with antibodies to GC and CNP (A,B); G_{D3} and GC (C,D) and single labelling on day 5 (E) and day 6 (F) with antibodies to CNP (E) and MBP (F). Mature oligodendroglia can be seen at day 4 expressing both GC and CNP (A,B). Immature, bipolar G_{D3}^+ progenitors can still be seen at day 6 amongst mature oligodendroglia (C,D). The mature cells have a characteristic elaborate process network and express the myelin components CNP (E) and MBP (F). Scale bar=40 μm.

18 h pulse. The G_{D3}^+ cells at this stage, now in serum-free medium, were largely immature, bipolar cells and again it was these cells that had incorporated BrdU. Less than 1% of GC^+ cells had incorporated BrdU (average of 527 GC^+ cells counted per coverslip; $n=5$) (Fig. 6B).

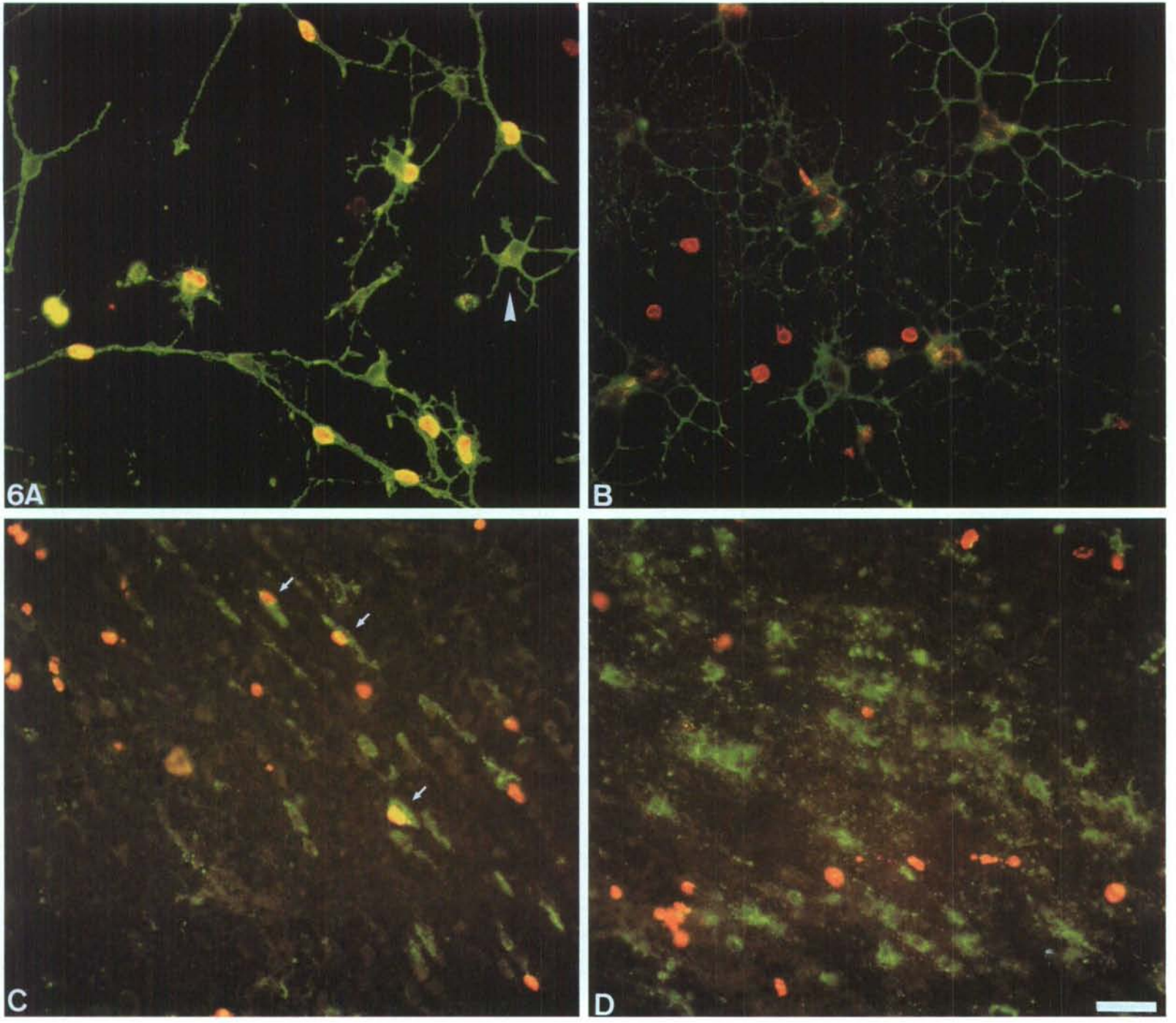


Fig. 6. Double immunofluorescent labelling of progenitor enriched secondary cultures on day 1 (A) and day 5 (B) and of postnatal day 7 rat forebrain sections (C,D) with antibodies to G_{D3} (A and C; fluorescein) and BrdU (rhodamine) and to GC (B and D; fluorescein) and BrdU (rhodamine). On day 1 of secondary culture many bipolar G_{D3}^+ progenitors can be seen to have incorporated BrdU after an 18 h pulse, whereas the more mature cells have not (A; arrowhead). On day 5 the numerous GC^+ cells are negative for BrdU although some cells appear weakly positive due to the background staining of cell bodies seen here (B). In cryostat sections taken after a 3 h pulse with BrdU G_{D3}^+ cells can clearly be seen to have incorporated BrdU (C; arrows) but GC^+ oligodendroglia have not (D). Scale bar=30 μm.

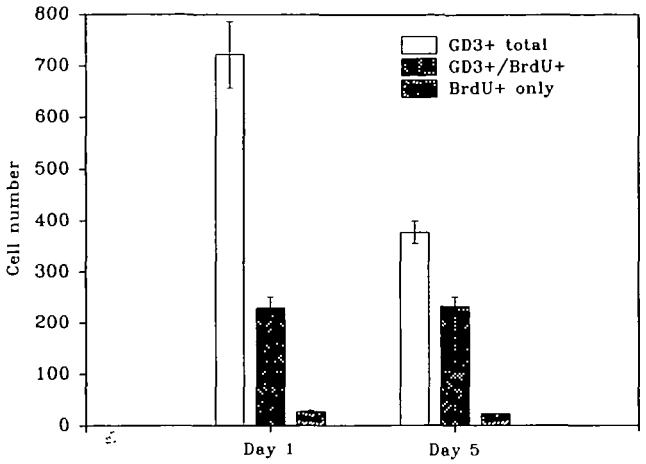


Fig. 5. Mitogenic profile of O-2A progenitor cells in secondary culture. Cultures were pulsed with BrdU (10 μM) for 18 h then immunostained. 20 fields (40×) were counted per coverslip. Numbers are means ±S.E.M. of 5–20 coverslips from 3–4 separate experiments.

Development of progenitors in serum containing culture

When the enriched progenitor population was maintained in medium containing 10% FCS, by 3 days in secondary culture the number of G_{D3}^+ cells also expressing GFAP had increased slightly (Fig. 7). G_{D3}^+ progenitor cells became multipolar and retained the expression of vimentin intermediate filaments. By the 6th day of secondary culture, the majority of G_{D3}^+ cells co-expressed both GFAP and vimentin and bore the stellate morphology of type-2-like astroglia. There was a sharp fall in the number of cells expressing G_{D3} alone. These cells were bipolar or of an immature stellate morphology. The number of GC^+ oligodendroglia remained at less than 5% of total cells throughout.

After 10 days in secondary culture, the more fibrous looking of the type-2-like astroglia had ceased to express G_{D3} and also vimentin. The increase in G_{D3}^-/$GFAP^+$ cells at this stage seen in Fig. 7 was partly due to the proliferation of contaminating type-1-like astroglia, which failed to survive in serum-free medium.

Analysis of the preprogenitor cell

It was clear from our data that the total number of cells stained with our panel of antibodies did not correlate with the total number of cells present as counted using phase-contrast optics (Fig. 8). This was especially noticeable at day 1 where approximately 30% of cells remained unstained. These cells were small and round and resembled the small apolar G_{D3}^+ cells seen at day 4–5. $Vimentin^+/G_{D3}^-$ cells seen at day 1 also had this morphology (Fig. 9). The development of these cells was studied by selectively killing G_{D3}^+ and GC^+ cells using complement-dependent cell lysis before plating the cells in secondary culture. The antigenic development of this cell population was followed over the next 2 days both in serum-free medium and serum-containing medium. The results are shown in Fig. 10. After complement lysis, cells were stained in suspension (day 0) and 44% of the cells were positive for vimentin, whereas only 6% were G_{D3}^+ and 4% were GC^+. Twenty four hours later the proportion of G_{D3}^+ cells had increased to 22% of total cells. By day 2, G_{D3}^+ cells accounted for 35% of total cells and GC was expressed in 11% of cells. The percentage of $vimentin^+/G_{D3}^-$ cells reduced after 1 day to 6% of total cells. Thus it appears that these vimentin+ cells become G_{D3}^+ O-2A progenitors. Newly arisen G_{D3}^+ cells were either small and apolar or slightly larger with a few short processes. Small classical bipolar progenitor cells could also be seen. Although the cells did not appear to divide to a great extent in SFM, preliminary results indicated that they could be induced to divide in

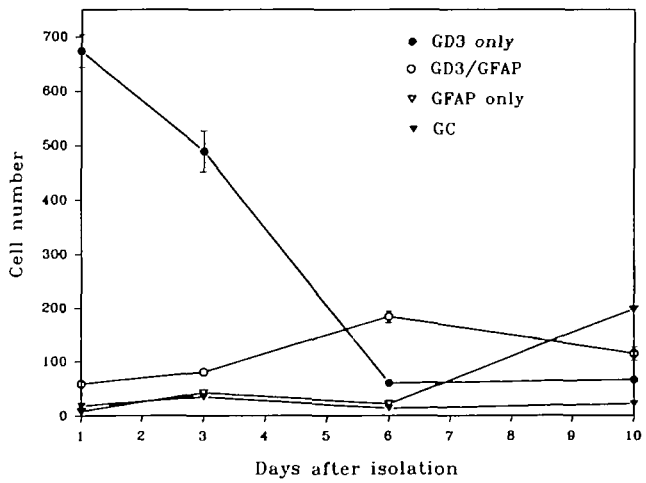


Fig. 7. Antigenic profile of O-2A progenitor enriched secondary cultures in serum-containing medium. Cells were maintained in medium containing 10% FCS and were immunostained on days 1, 3, 6 and 10 of secondary culture. 20 fields (40×) were counted per coverslip. Numbers are means ±S.E.M. of 3–14 coverslips from 2 to 3 separate experiments.

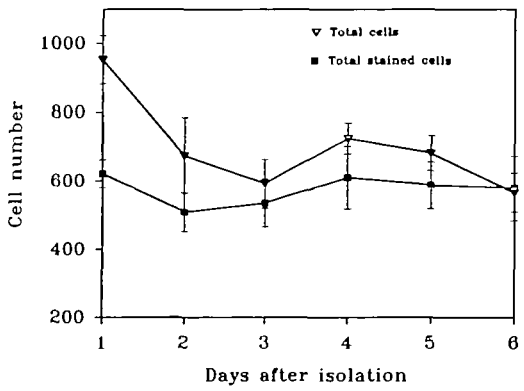


Fig. 8. Comparison between total cell number, as determined by phase-contrast optics and total number of immunostained cells. 20 fields (40×) were counted on each coverslip. Numbers are means ±S.E.M. of 7–39 coverslips from 2 to 5 separate experiments.

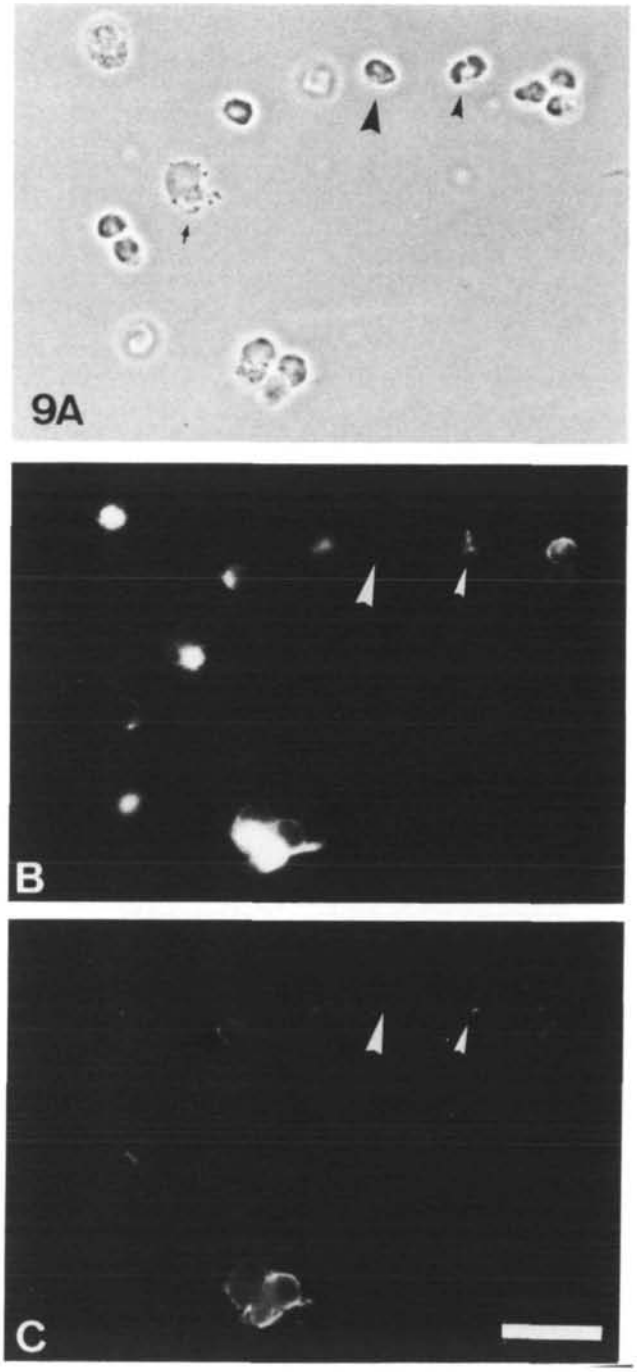


Fig. 9. Double immunofluorescent labelling of preprogenitor cultures 1 day after elimination of G_{D3}^+ and GC^+ cells by antibody-dependant complement lysis with antibodies to vimentin (B) and G_{D3} (C). The same field is shown using phase-contrast optics (A). Both G_{D3}^-/Vim^- (large arrowhead) and G_{D3}^-/Vim^+ (small arrowhead) preprogenitors can be seen, as well as a Vim^+ cell which has already begun to express G_{D3}. Several Vim^+ ameboid microglia can clearly be distinguished by their characteristic appearance in phase contrast (arrow). Scale bar=30 μm.

type-1-like astroglial conditioned medium (unpublished observations).

We were unable to culture this preprogenitor population of cells for more than 2 days of secondary

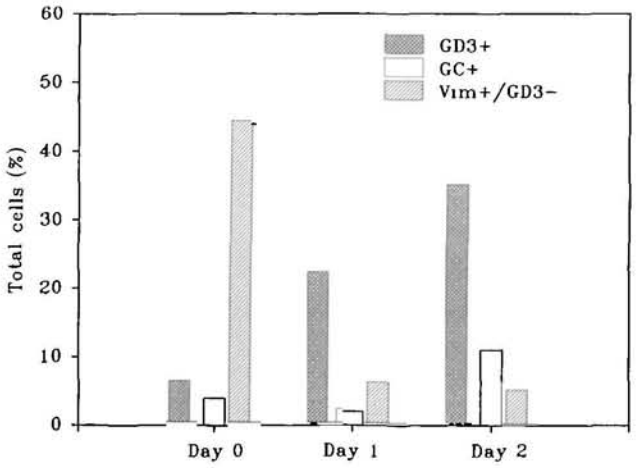


Fig. 10. Antigenic profile of cultures after treatment with complement and LB_1 and GC antibodies. Cells were immunostained in suspension immediately following treatment and on coverslips 1 and 2 days afterwards. Numbers are percentages of total cells. At least 100 cells (cell suspensions) or 20 fields (40×) were counted on 2–8 coverslips from 3 separate experiments. SEM is less than 20% of mean in all cases. Increases in numbers of G_{D3}^+ cells are statistically significant to $P<0.05$ (Student's t-test).

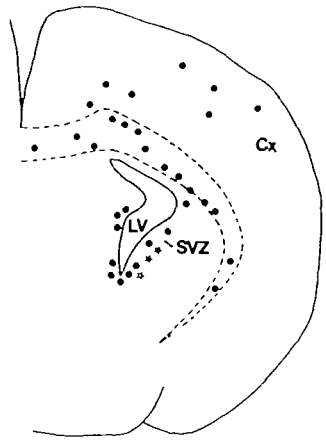
culture. It is likely that the presence of other factors or cell types is required for their survival. When cultured in serum-containing medium, cell survival improved but no GC^+ cells were seen. $G_{D3}^+/GFAP^+$ stellate astrocytes could be seen after 5 days in secondary culture (data not shown).

Around half the population remaining after complement lysis with G_{D3} and GC antibodies was $vimentin^-/GD3^-/GC^-$. These cells also failed to express GFAP, and were not labelled by the mAb OX42. These cells did not express GAP-43, eliminating the possibility that they were immature neurones. They had the small round morphology of the $vimentin^+/G_{D3}^-$ cells. The identity and growth requirements of these cells is at present under investigation.

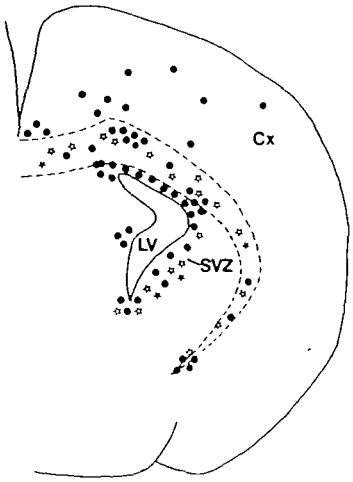
Development of progenitors and oligodendroglia in vivo

(a) *Embryonic day 19–20 (Fig. 11A)*

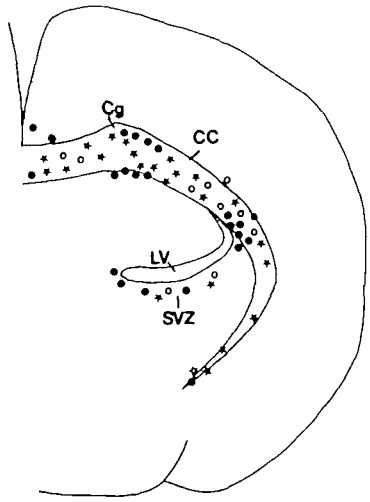
There were large numbers of G_{D3}-expressing cells throughout the sections at this stage. They were concentrated mainly in the formative subcortical white matter where they appeared either round with no apparent processes, or thinner and more bipolar. These cells were also seen at the lower edge of the lateral ventricle and medially between the two ventricles. The large subventricular zone (SVZ) of the lateral ventricle present at this age consisted of many small round, tightly packed cells. Those nearest the ventricle contained vimentin-positive intermediate filaments but vimentin expression appeared to decrease with increasing distance from the ventricle. Conversely, cells nearest the ventricle did not express G_{D3} but further into the SVZ several G_{D3}^+ cells were seen packed between the $vimentin^+$ cells. Only rarely did a cell



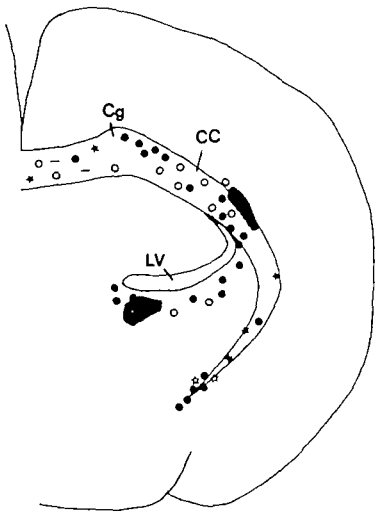
11A. EMBRYONIC DAYS 19–20



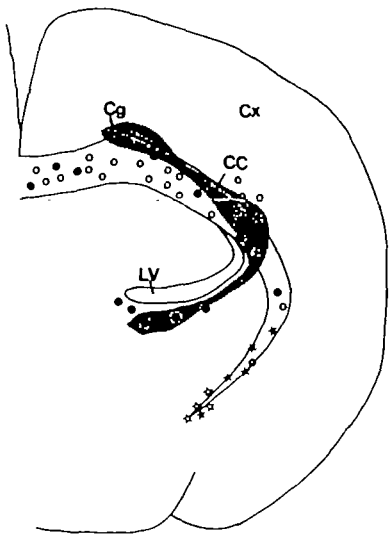
B. POSTNATAL DAYS 0–2



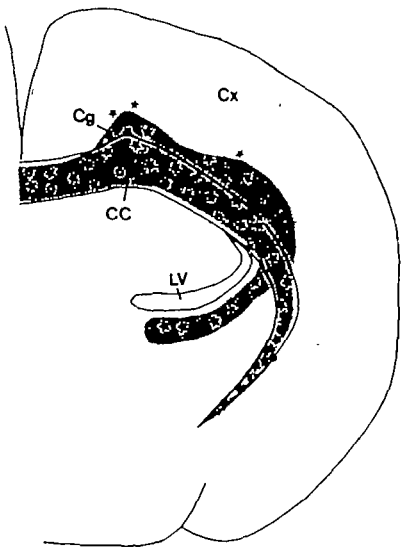
C. POSTNATAL DAYS 3–4



D. POSTNATAL DAYS 6–8



E. POSTNATAL DAYS 9–12



F. POSTNATAL DAYS 15–20

Fig. 11. Diagrammatic representation of antigen distribution in the developing corpus callosum. Diagrams were constructed from immunostained coronal 5 μm cryostat sections from rat forebrain using symbols as follows: ●, G_{D3}; *, G_{D3}/GC; ☼, GC; ○, GC/CNP; ✱, MBP; – myelin segments; □, densely myelinated areas; CC, corpus callosum; LV, lateral ventricle; Cx, cortex; Cg, cingulum; SVZ, subventricular zone.

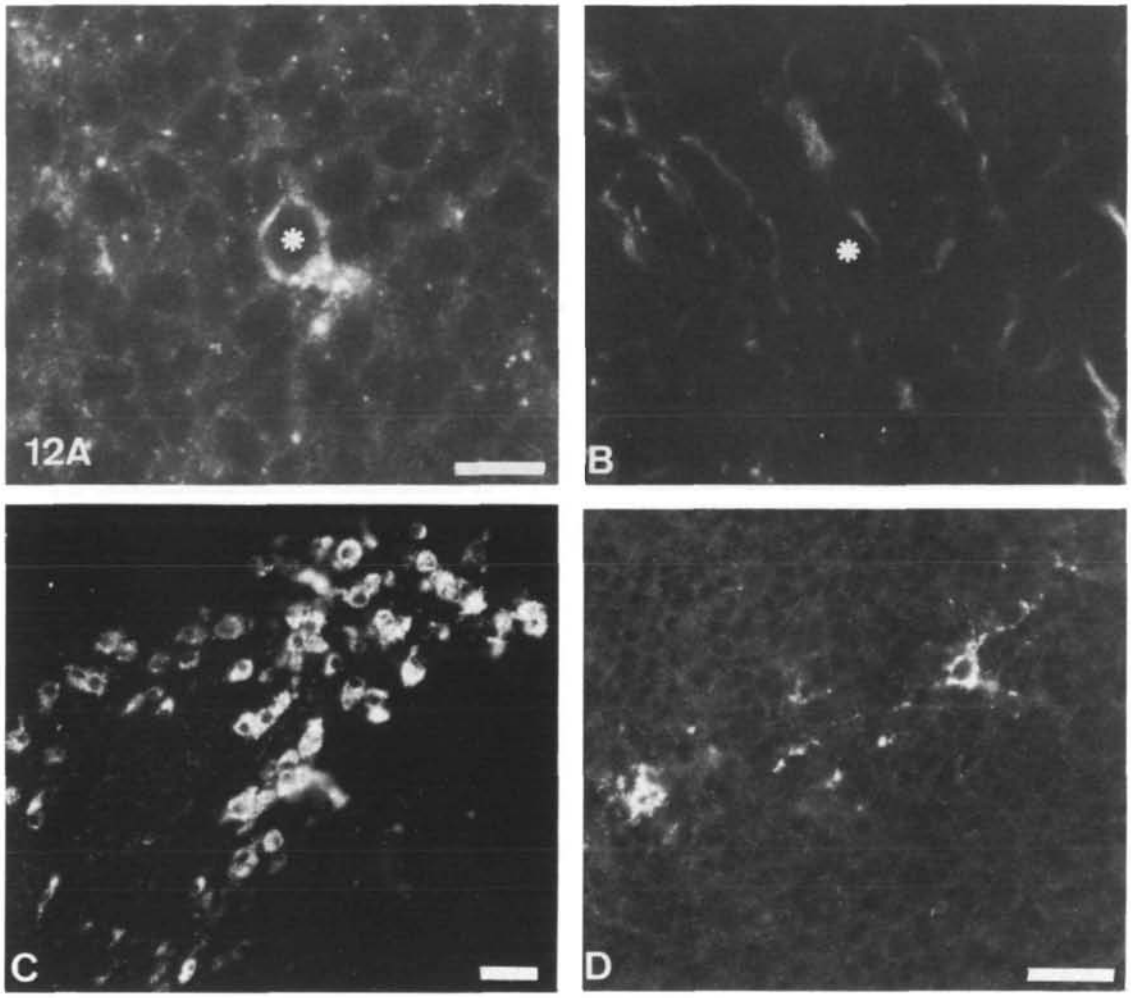


Fig. 12. Double immunofluorescent labelling of embryonic day 19 (A,B) rat forebrain sections with antibodies to G_{D3} and vimentin and single labelling of postnatal day 0 (C) and 2 (D) rat forebrain sections with antibodies to G_{D3} (C) and GC (D). A double labelled G_{D3}^+/vimentin$^+$ cell is seen amongst the tightly packed cells of the SVZ (A,B; asterisk). Large numbers of G_{D3}^+ cells lie around the ends of the lateral ventricle at these early stages (C). GC$^+$ cells are clearly seen in the SVZ at P0 (D). Scale bars (A,B)=10 μm; (C,D)=30 μm.

appear to co-express vimentin (Fig. 12A,B), although the resolution of the light microscope is not adequate to be sure of this.

G_{D3}^+ cells could also be seen radiating out from the formative white matter into the cortex. It is possible that these may be neuronal precursors of neuroectodermal origin as these express G_{D3} at an early stage, although they cease to do so postnatally. In the cerebellum, the dividing granule neurones of the external granule cell layer cease to express G_{D3} prior to their migration into the internal granule layer (Reynolds and Wilkin, 1988). Vimentin$^+$ cells were seen in the outer regions of the cortex, often with a process attached to the pial surface, characteristic of radial glia (Hirano and Goldman, 1988). A few of these cells also expressed G_{D3}.

A few GC$^+$ cells were seen at this age. They resided in the lower SVZ and were small and of immature morphology. Occasional G_{D3}-expressing cells in the inferior formative white matter were also faintly GC$^+$. The morphology of these cells, small with a few delicate processes, indicates that they are immature in nature and have probably recently arisen from glial progenitor cells. This is confirmed by the fact that they failed to express CNP or MBP, suggesting a newly differentiated cell.

(b) *Postnatal day 0–2 (Fig. 11B)*

Large numbers of G_{D3}^+ cells were still present during this period. The majority were the large round cells seen earlier, with variable amounts of cytoplasm, resembling those seen by Goldman and colleagues (Levine and Goldman, 1988*a*,*b*,*c*). These cells were seen predominantly in clusters at either end of the lateral ventricle, spread along the SVZ and adjacent white matter, in the cingulum and at the lower tips of the formative white matter (Fig. 12C). Others, interspersed between these cells had the smaller, more elongated cell bodies and sometimes appeared to extend one or two processes from either or both ends. Further G_{D3}^+ cells were seen in the grey matter

immediately overlying the cingulum and spreading outwards towards the pial surface. These cells tended to be more compact in appearance than those seen in formative white matter. A cluster of G_{D3}^+ cells were also seen in the grey matter immediately over the most medial section of the subcortical white matter. Fewer cells than previously were seen in the cortical grey matter, presumably reflecting the maturity of neurones, which had now ceased to express G_{D3}.

GC^+ cells were seen within clusters of G_{D3}^+ cells, a few of which coexpressed both antigens. These double-labeled cells had an immature morphology with only one or two short processes. Cells of a more mature morphology expressing GC had a small cell body and several processes. These were seen in the SVZ, in neighbouring white matter, and in the inferior regions of the formative white matter tract (Fig. 12D). These more-mature-looking cells also expressed CNP, although some only faintly. A rare MBP^+ cell was seen by postnatal day 2 but most GC cells remained MBP^- at this stage. Vimentin intermediate filaments were again seen in abundance in the SVZ and formative white matter but, although G_{D3}^+ cells resided in the same areas, no cells positive for both antigens could be detected. The SVZ cells were less compact at this stage and the vimentin processes were more elaborate, making it harder to distinguish to which cell they belonged. It was impossible, at the light microscopic level, to localise unequivocally both antigens to the same cell. This was also the case for GFAP intermediate filament processes, which often surrounded the G_{D3}^+ cell body, but did not appear to be a part of the same cell.

(c) *Postnatal day 3–4 (Fig. 11C)*

At this age, G_{D3}^+ cells were confined almost exclusively to clusters in the cingulum (Fig. 13A), at either end of the lateral ventricle and in the formative white matter adjacent to the lateral ventricle and at its lower tips. Their numbers were reduced compared to previous ages. The cells were still mostly large and round with no apparent processes although some were elongated in shape. Cells co-expressing G_{D3} and GC were apparent in several clusters of G_{D3}^+ cells, notably in the SVZ and neighbouring subcortical white matter (Fig. 14A,B). Again, G_{D3}^+ cells were seen just outside the region of vimentin immunoreactivity lining the lateral ventricle (Fig. 14C,D).

GC^+ oligodendroglia were now spread throughout the formative white matter. They had a range of morphologies from small round cells with several processes in the cingulum (Fig. 13B) to cells with an elaborate process network in the subcortical white matter and in the SVZ. Most GC^+ cells expressed CNP at this stage (Fig. 13C) with the exception of the round cells within clusters of G_{D3}^+ cells. These are presumably less mature cells, which had recently begun to differentiate but were yet to express CNP. The GC^+ cells with more elaborate process networks also expressed MBP thus confirming their maturity (Fig. 13D).

GFAP staining was more intense than previously, especially in the cingulum where a mass of $GFAP^+$ fibres radiated out into the grey matter. These fibres were also positive for vimentin. Again, we were unable to determine if the vimentin$^+$/$GFAP^+$ processes belonged to cells expressing G_{D3}.

(d) *Postnatal day 6–8 (Fig. 11D)*

G_{D3}^+ cells could be found in large numbers throughout the corpus callosum. The large round cells remained confined to the cingulum, white matter adjacent to the lateral ventricle and callosal tips (Fig. 15A). Smaller, bipolar cells were seen elsewhere in the corpus callosum (Fig. 15C).

The number of GC^+ cells had increased dramatically by this stage, most of them also expressing CNP. Cells were invariably process-bearing throughout the formative white matter. Cells were often seen in pairs or rows, characteristic of interfasicular oligodendrocytes, especially in the central corpus callosum (Fig. 15B). The number of cells expressing MBP had also increased, most of which lay in the formative white matter adjacent to the lateral ventricle and in neighbouring grey matter at 6 days postnatal but were more widespread throughout the corpus callosum by the 8th postnatal day (Fig. 15D). Short lengths of MBP^+ myelin sheaths were visible, notably in regions inferior and lateral to the SVZ.

(e) *Postnatal day 9–12 (Fig. 11E)*

This period marked a rapid increase in myelin formation. MBP^+ sheaths were seen running down the corpus callosum from the cingulum to the lateral ventricle, and in cross section in the cingulum. These sheaths were also positive for CNP and GC (Fig. 16A,B). Cell bodies positive for CNP (Fig. 16C), MBP (Fig. 16D) and GC were seen amongst these sheaths. GC^+/CNP^+ cells were also seen in the more inferior regions of the corpus callosum, where MBP^+ cells were rare.

G_{D3}^+ cells were seen up to the 12th postnatal day, but in decreasing numbers. A few large round cells were seen in the cingulum together with some small bipolar cells in the white matter.

(f) *Postnatal day 15–20 (Fig. 11F)*

By this stage, regions of CNP^+/MBP^+ myelin sheaths could be seen throughout the corpus callosum. Oligodendroglial cell bodies expressed CNP but not MBP in these regions (Fig. 17A). Cross sections of myelinated axons were packed into the cingulum and extended out into the neighbouring grey matter (Fig. 17B). A meshwork of myelinated axons could be seen in the cortical grey matter adjacent to the corpus callosum, which extended out towards the pial surface with time (Fig. 17E). GC^+/CNP^+ cell bodies could clearly be seen in regions of grey matter near the pial surface that were yet to be extensively myelinated. They could be seen extending processes to short myelin segments (Fig. 17C and D). By this stage no G_{D3}^+ cells were seen.

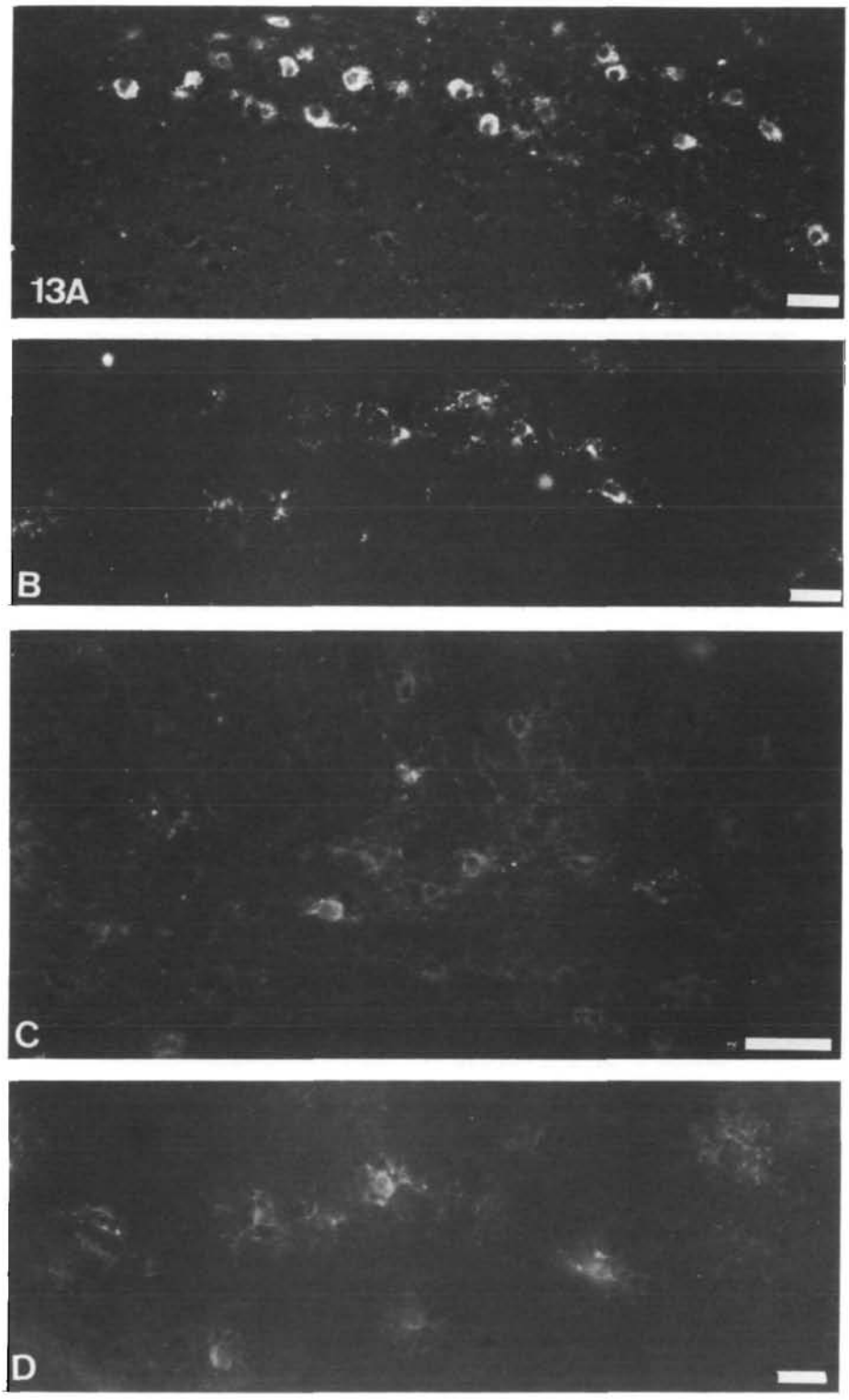


Fig. 13. Single immunofluorescent labelling of postnatal day 3 (A and B) and 4 (C and D) rat forebrain sections with antibodies to G_{D3} (A), GC (B), CNP (C) and MBP (D). In the cingulum, large round G_{D3}^+ cells (A) coexist with maturing GC^+ oligodendroglia (B). The latter increase in number, become more elaborate and express CNP (C). Mature oligodendroglia expressing MBP can be seen in the developing white matter (D). Processes can be seen extending to axons. Scale bars=30 μm.

BrdU incorporation in vivo

BrdU was used to label proliferating cell types *in vivo*. Two injections of BrdU were given during a 3 h pulse to pups of various ages. At 24 h after birth (P1), some G_{D3}^+ cells were seen to have incorporated BrdU. These resided mainly near the top of the lateral ventricle or in the cingulum. None of the few GC^+ cells present at this stage were double labeled for BrdU. At P4 no G_{D3}^+ or GC^+ cells were seen double labeled for BrdU. At P7 a significant proportion of G_{D3}^+ cells had incorporated BrdU (Fig. 6C). These were found within clusters of large round cells in the cingulum and in the corpus callosum adjacent to the lateral ventricle. Occasionally smaller bipolar G_{D3}^+ cells in the white

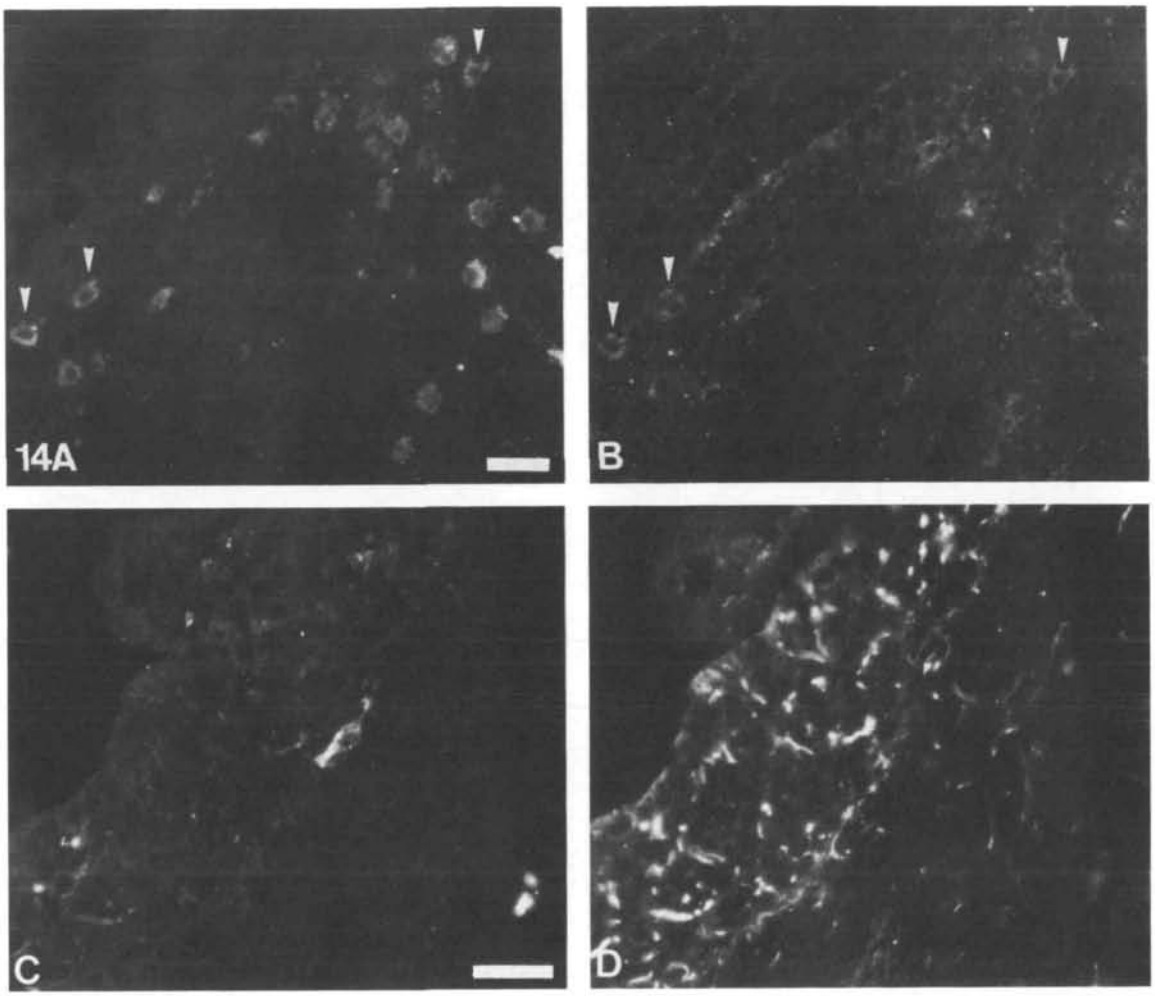


Fig. 14. Double immunofluorescent labelling of postnatal day 3 (A,B) and 4 (C,D) rat forebrain sections with antibodies to G_{D3} and GC (A,B) and G_{D3} and vimentin (C,D). Several cells expressing both antigens can be seen in this group of progenitor cells in the SVZ (arrowheads). Cells positive for either G_{D3} or GC can also be seen. The cells lining the lateral ventricle are vimentin$^+$ but G_{D3}^-. A G_{D3}^+/vimentin$^-$ cell is seen on the edge of the area of vimentin$^+$ cells. Scale bars=30 μm.

matter between the cingulum and the lateral ventricle had also incorporated the label. Of the numerous GC$^+$ cells seen at this age, only one or two were ever seen to have incorporated BrdU (Fig. 6D). By P12 the number of G_{D3}^+ cells had dramatically reduced. None were double labeled with BrdU. No GC$^+$/BrdU$^+$ cells were seen at this age. At all ages studied, many of the small compact cells of the SVZ lining the lateral ventricle were BrdU$^+$. Occasionally, one of these further away from the ventricle was G_{D3}^+.

In a separate experiment, postnatal day 7 pups were given a 6 h pulse of BrdU. This resulted in a larger number of G_{D3}^+ cells incorporating BrdU compared to the 3 h pulse. No GC$^+$ cells were double labelled during the longer pulse.

Discussion

We have established a culture system enriched in G_{D3}-expressing O-2A-like glial progenitor cells which are poised to differentiate into oligodendroglia when cultured in a serum-free chemically defined medium. These cells then go on to express myelin components, form elaborate process networks and finally myelin-like membranes. When cultured in the presence of 10% foetal calf serum, they differentiate into type-2-like astroglia similar to those derived from the neonatal optic nerve (Raff *et al.* 1983). G_{D3}-expressing glial progenitors from neonatal rat forebrain thus behave similarly to the O-2A progenitors of the optic nerve. A study of glial progenitors from the rat cerebrum using A2B5 antibody reactivity as a marker for O-2A cells also showed a close resemblance between these cells and those derived from the optic nerve (Behar *et al.* 1988). However, it is not possible to sustain a comparison between *in vitro* and *in vivo* data using A2B5 due to its low specificity in tissue sections (Miller *et al.* 1989).

We have summarised in diagrammatic form (Fig. 18) our data obtained from both *in vitro* and *in situ* experiments from this and other studies, to compare the

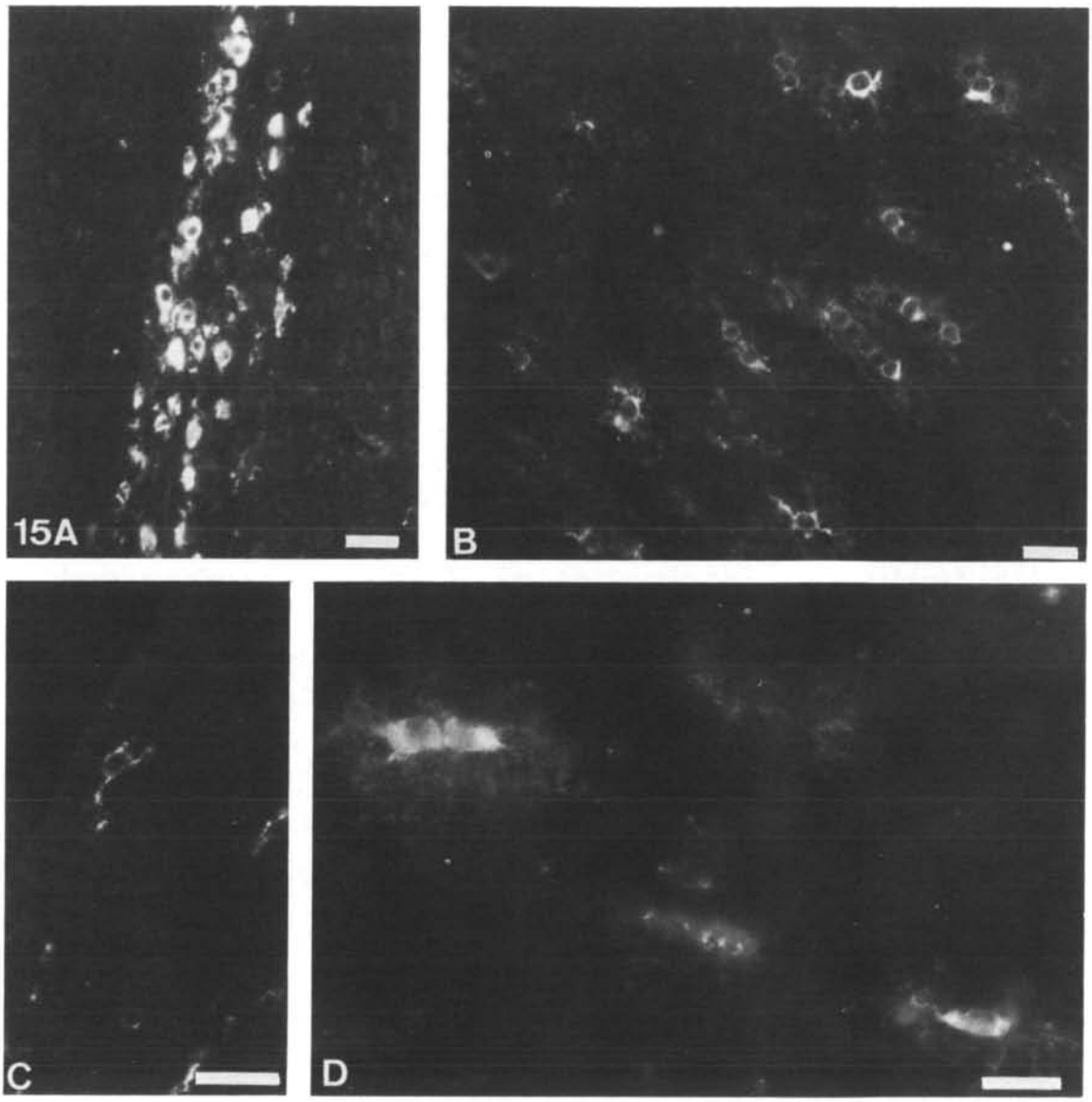


Fig. 15. Single immunofluorescent labelling of postnatal day 7 (A,B and C) and 8 (D) rat forebrain sections with antibodies to G_{D3} (A and C), GC (B), and MBP (D). Large round G_{D3}^+ cells are seen in clusters, here in the inferior subcortical white matter (A). More delicate, apparently bipolar G_{D3}^+ cells are also seen, here just outside the lateral ventricle (C). Interfasicular oligodendroglia can be identified in the corpus callosum expressing both GC (B) and MBP (D). Scale bars (A,B,C)=30 μm; (D)=20 μm.

overall developmental pattern that forebrain glial progenitors follow when they progress along the oligodendroglial pathway.

In addition to the major population of G_{D3}-expressing progenitors isolated from the O-2A enriched glial cultures, we have also described a cell type that we have termed a preprogenitor. These cells expressed no G_{D3} whilst in the mixed cultures or when isolated, but rapidly began to express G_{D3} followed by GC when cultured in serum-free medium. BrdU studies revealed that in the isolated preprogenitor cultures maintained in SFM, only 10% of G_{D3}^+ cells were proliferating during an 18 h pulse between days 0 and 1 after isolation. Hence the increase in G_{D3}^+ cells could not be primarily due to the proliferation of residual G_{D3}^+ cells left after complement lysis. Approximately 40% of these G_{D3}^- preprogenitors expressed vimentin filaments. Grinspan *et al.* (1990) recently reported a vimentin$^+$ cell in cultures of cerebral white matter, which may give rise to A2B5$^+$ O-2A progenitors under the influence of PDGF, but not in its absence, and only after 10 days in mixed cell cultures. It is proposed that those G_{D3}^- cells in our cultures that failed to express vimentin are at an earlier stage of development and will go on to express both vimentin and G_{D3}. *In vivo*, the small tightly packed cells lining the lateral ventricle, forming the subventricular zone, were vimentin$^+$ but G_{D3}^-. These cells are likely to be the source of the preprogenitors in our cultures. G_{D3}^+ cells were seen further from the lateral ventricle where the occasional G_{D3}^+vimentin$^+$

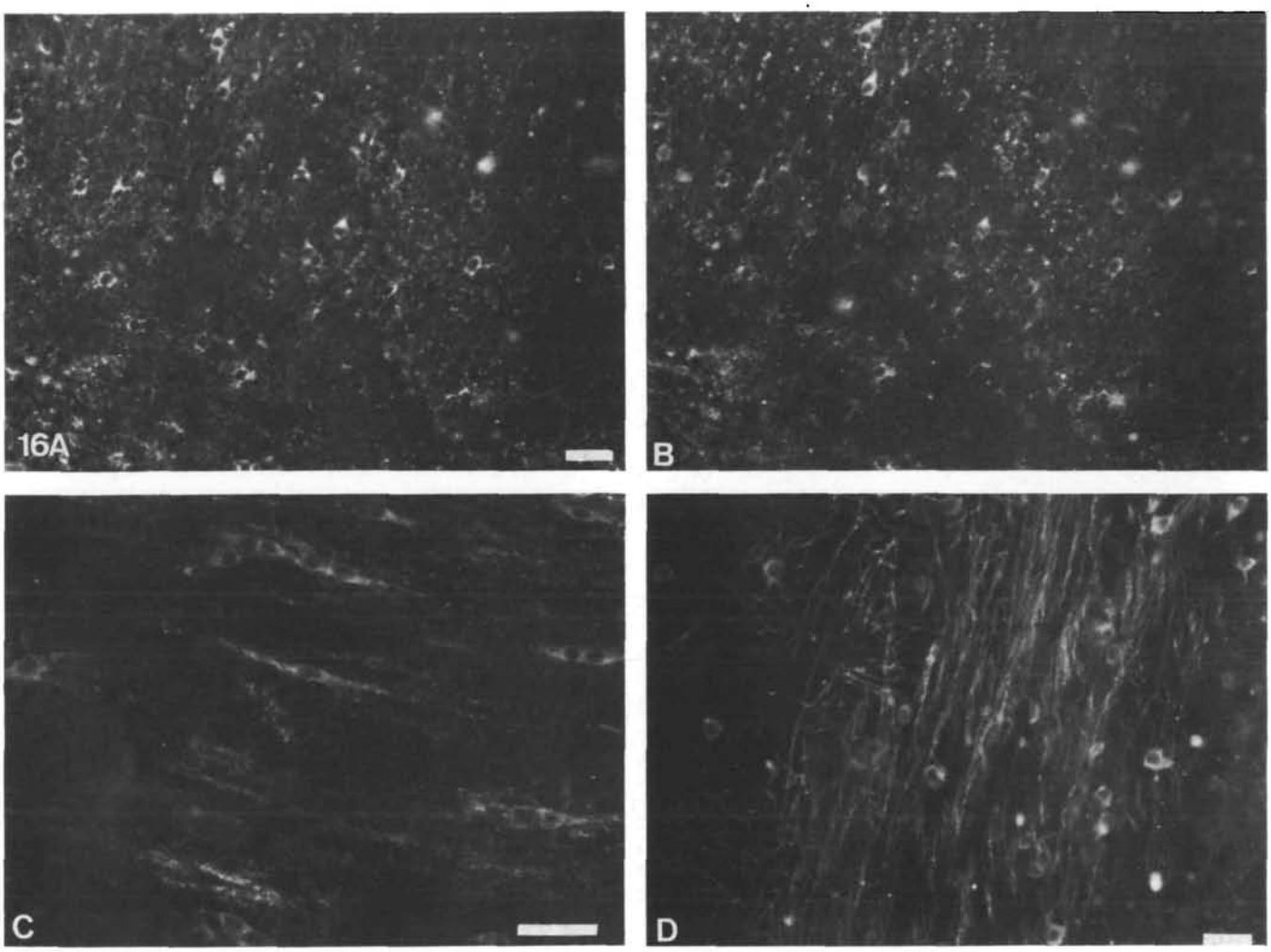


Fig. 16. Double immunofluorescent labelling of postnatal day 9 rat forebrain sections with antibodies to GC and CNP (A,B) and single labelling with antibodies to CNP (C) and MBP (D). All oligodendroglia in the white matter expressed both GC and CNP at this age. Shown here is the cingulum and adjacent white matter (A,B). CNP^+ cell bodies can clearly be identified in the central corpus callosum (C) where there is as yet little myelin. In the regions of the subcortical white matter adjacent to the lateral ventricles, MBP^+ myelin sheaths can be seen as well as MBP^+ cell bodies (D). Scale bars=30 μm.

cell was seen. This suggests that the $vimentin^+$ cells migrate out of the subventricular zone before becoming $G_{D3}{}^+$. These results differ from those of Levine and Goldman (1988*b*) who did not observe $G_{D3}{}^+$/$vimentin^+$ cells close to the ventricle. It may be that SVZ cells *in vivo* cease to express vimentin almost immediately on acquiring G_{D3} and so cells expressing both antigens are rarely seen. This is not the case *in vitro* where $G_{D3}{}^+$ progenitors continue to express vimentin until the acquisition of GC. It is possible that the failure to colocalise both antigens to the same cell was due to insufficient sensitivity of the light microscopic immunochemical technique. However, Levine and Goldman (1988*c*) failed to find intermediate filaments in cells of the cingulum stained with an antibody to G_{D3} at the electron microscope level. It may be that the time course of vimentin expression is altered in culture. This temporal elongation of antigen expression *in vitro* is also seen for the G_{D3}/GC transition. Cells positive for both G_{D3} and GC are found only in small numbers in tissue sections but can account for up to 20 % of GC^+ oligodendroglia *in vitro*. G_{D3} appears to be present on the developing oligodendroglia for an extended period in culture and cells of a highly differentiated morphology are often seen.

The glial progenitors in our cultures, when maintained in serum-free medium, began to differentiate after 1 day in secondary culture. They became post mitotic and multipolar, although they did not begin to express GC for another 24 h. Differentiation then proceeded rapidly, peaking only 48 h after the first appearance of GC (Fig. 2). The differentiation of the O-2A progenitor cells *in vivo* occurred over a longer period of time, increasing numbers of GC^+ cells were seen from P2 to P7. This is clearly due to the fact that glial progenitors *in vivo* are a heterogeneous population with respect to their stage of development. This will provide a steady production of oligodendroglia

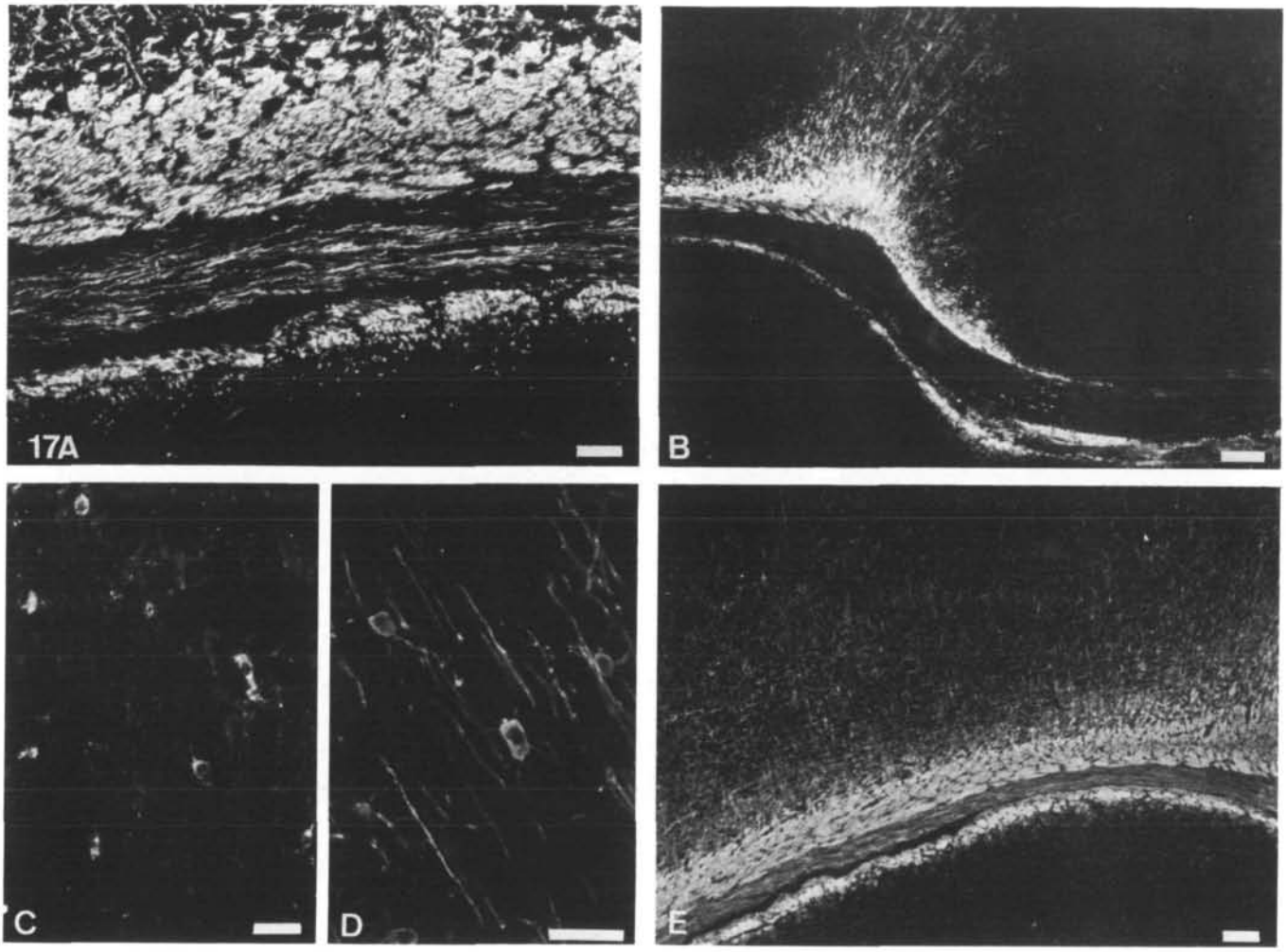


Fig. 17. Single immunofluorescent labelling of postnatal day 17 (A and B) and 21 (C,D and E) rat forebrain sections with antibodies to MBP (A,B and E); GC (B) and CNP (C). MBP^+ myelin sheaths can now be seen longitudinally in the white matter tract and in cross section in the cingulum but MBP is no longer expressed in oligodendroglial cell bodies (A and B). Isolated GC^+ and CNP^+ cell bodies can be seen in the cortical grey matter extending processes to myelinated axons (C and D). By P21 myelin is now more abundant and myelination has spread well into the cortical grey matter (E). Scale bars=30 μm.

throughout the first postnatal week. In our cultures, cells have been held in an undifferentiated state for 12 days in primary culture and so are all primed to differentiate upon the withdrawal of PDGF, in the form of type-1-like astroglia (Noble *et al.* 1988), and the removal of serum inhibitory factors (Bologa *et al.* 1988).

GC^+ cells were seen in the SVZ as early as E19. Other reports suggest that oligodendroglia arise at around the time of birth both in the cerebellum (Reynolds and Wilkin, 1988) and the optic nerve (Miller and Raff, 1985). In studies on cell suspensions from whole embryonic and postnatal rat brain, GC^+ oligodendroglia were not detected until 2–3 days after birth (Abney *et al.* 1981). However, this technique may not have been sufficiently sensitive to detect the small number of GC^+ cells seen here. A recent report by Bansal and colleagues (Bansal *et al.* 1989) has thrown doubt on the specificity of the anti-GC antibody, R-mAb, used here. It appears that the antibody reacts with both galactocerebroside and sulphatide and labels developing oligodendroglia in culture at a slightly earlier stage than another anti-GC monoclonal antibody, O1. This may account for the early appearance of GC^+ cells in tissue sections here although no comparison between R-mAb and O1 has yet been made *in vivo*.

The number of O-2A progenitors fell steadily over the first 4 days in secondary culture, after which their number remained stable. In an 18 h pulse between days 4 and 5, 62% of the progenitors incorporated BrdU. This compares with only 32% of progenitors incorporating the label in a similar pulse between days 0 and 1. It appears that not all the progenitors are primed to differentiate immediately and that those remaining are dividing to renew progenitor numbers, even in the total absence of FCS. It is possible that the population of cells undergoing division at D0/D1 of secondary culture is the same population that is dividing at D4/D5. Hence differentiation of non-mitotic progenitors would take

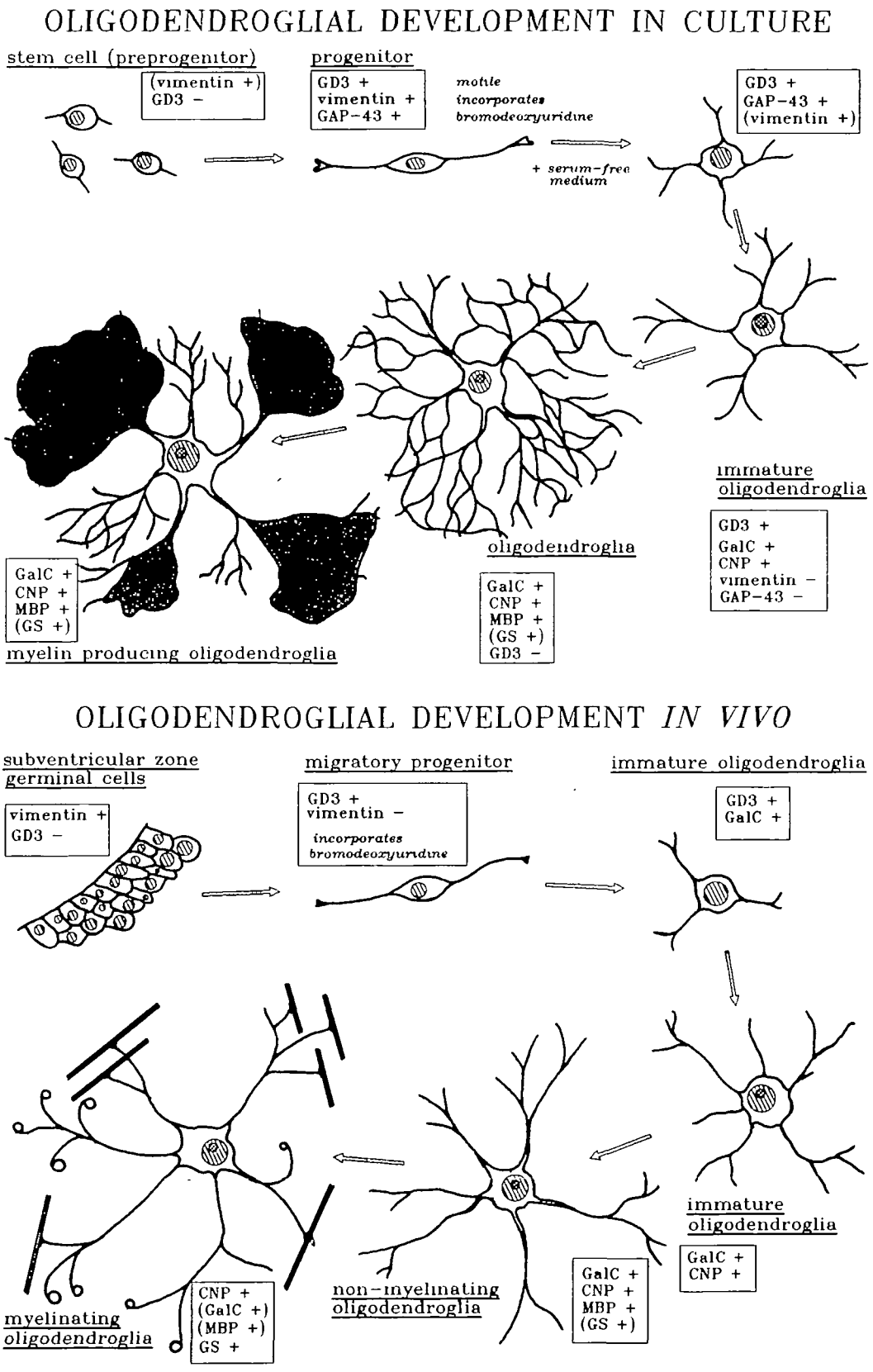


Fig. 18. Diagrammatic summary of cell morphology and antigenic phenotype of O-2A progenitor cells and oligodendroglia during development *in vitro* and *in vivo*.

place on days 0–4, leaving a lārger percentage of G_{D3}^+ cells in the mitotic phase. These may form a self-renewing population of cells akin to the unipolar optic nerve adult progenitor described by Wolswijk and Noble (1989). However, although some mitotic cells were unipolar, they were not exclusively so, and some unipolar cells were seen at all stages of secondary culture. Gard and Pfeiffer (1989) reported a population of $O4^+$ cells that failed to differentiate after 10 days in culture. However, these were not dividing and so are unlikely to represent a self-renewing population. A further report has been made of a slowly proliferating cell of the oligodendroglial lineage (Dubois-Dalcq, 1987). This study of optic nerve progenitors showed a multipolar, $O4^+$ cell which slowly divides but appears to differentiate in the presence of insulin.

The apparent increase in mitogenesis of the O-2A progenitor population after the first wave of differentiation may reflect the immature nature of a population recently derived from preprogenitor cells. The presence of small round G_{D3}^+ cells at 4–5 days in secondary culture would seem to confirm this. *In vitro* BrdU studies indicate that it is only the immature, bipolar progenitor cells that undergo division, a cell type that is predominant at this stage in secondary culture, hence a high labelling index for G_{D3}^+ cells would be expected. This would also account for the low labelling index of GC^+ cells, as progenitor cells appear to drop out of the cell cycle around 24 h before they begin to express GC. This was also shown to be the case for optic nerve progenitors (Lillien and Raff, 1990). This would also explain the finding of Gard and Pfeiffer (1989) that only 2% of $O4^+$ cells in their cultures were dividing during the first 18–36 h in culture. O4 labels glial progenitors just before they are about to differentiate into oligodendrocytes but does not label the immature, bipolar progenitor cells (Gard and Pfeiffer, 1990; Hardy and Reynolds, unpublished observations).

The *in vivo* situation shows similarities with that *in vitro* with respect to the presence of G_{D3}^+ cells after the first wave of differentiation. *In vivo*, the number of O-2A progenitors appears to fall between P4 and P5 and then increase again up to P7. BrdU incorporation studies showed no labelling of G_{D3}^+ cells at P4, but at P7 G_{D3}^+ cells were seen to have incorporated BrdU over a 3 h period. It would appear that O-2A progenitor division is occurring at P7 ready for the production of oligodendrocytes needed for the large-scale myelination that occurs at postnatal days 10–15.

Our studies with BrdU both *in vivo* and *in vitro* indicate that the G_{D3}^+ O-2A progenitor is mitotic in its immature form whereas the immature GC^+ and more mature $GC^+/CNP^+/MBP^+$ oligodendroglia is not. The occasional GC^+ cell could be seen to have incorporated BrdU in our experiments both *in vivo* and *in vitro* but this was not usually the case. This confirms our previous *in vivo* study using ^{3}[H]thymidine in cryostat sections of the cerebellum (Reynolds and Wilkin, 1991) in which G_{D3}^+ O-2A progenitors incorporated the label but GC^+ oligodendroglia did not. Our results in the forebrain suggest that it is the O-2A progenitor and probably its precursor that divides during development. This is in agreement with a recent study by Gard and Pfeiffer (1990) using A2B5, O4 and GC labelling. The large numbers of $BrdU^+$ cells seen lining the lateral ventricle where the small round $vimentin^+/G_{D3}^-$ putative preprogenitors would be expected to reside would suggest that these are a highly mitotic population. Their failure to divide in culture may be the result of the absence of an appropriate signal. The lack of substantial numbers of $G_{D3}^+/BrdU^+$ cells around the SVZ suggests that the O-2A progenitors do not divide exclusively around the ventricle and then migrate to their destination, but that they can divide whilst migrating and/or at their final destination before differentiating into GC-expressing oligodendroglia.

G_{D3}^+ cells were rarely seen *in vivo* after 12 days postnatal. It is possible that detection of these cells in white matter is hampered by dense areas of myelin. GC^+ cell bodies were also difficult to locate in these regions. Evidence exists for the persistance of an O-2A progenitor cell into adulthood. The adult O-2A progenitor found in optic nerve cultures has been shown to coexist with perinatal forms as early as postnatal day 7 (Wolswijk *et al.* 1990). ffrench-Constant and Raff (1986) also reported an O-2A progenitor cell derived from adult rat optic nerve and suggested that these cells may be produced continually from pre-O-2A progenitor cells. However, an adult progenitor cell has yet to be demonstrated *in vivo* and therefore its antigenic phenotype *in vivo* is unclear, although from cell culture data it would be expected to express G_{D3}.

Expression of the myelin protein CNP closely followed that of GC both *in vivo* in cerebellum (Reynolds and Wilkin, 1988) and *in vitro* (Gard and Pfeiffer, 1989; Knapp *et al.* 1988). CNP^+/GC^- cells were seen *in vitro* but not *in vivo*, possibly as a result of the altered time span of antigenic expression *in vitro*. MBP expression closely followed that of GC and CNP *in vitro* but not *in vivo*. As in cerebellum (Reynolds and Wilkin, 1988, 1991), the first MBP^+ cell was seen 2–3 days after the first GC^+ cell in sections but by P7–8 almost all GC^+ cells were also MBP^+. Gard and Pfeiffer (1989) reported a 4–5 day lag between the appearance of GC and that of MBP in culture. These differences are likely to be due to different culture conditions, especially the use of different culture media and isolation techniques. Interestingly, earlier work in our laboratory revealed a 3–4 day lag between GC and MBP expression in cultures, but these contained a high proportion of contaminating microglia. These were seen to have a mitotic effect on the progenitor cells and may have delayed differentiation. *In vivo*, MBP is not seen in the cell bodies of myelinating oligodendroglia (Fig. 15A) in concordance with our previous study in the cerebellum (Reynolds and Wilkin, 1988). In culture, MBP was present in the cell body of all mature oligodendroglia, suggesting that these cells are primed to myelinate axons and it would be expected that co-culture with neurons would result in myelination and a corresponding decrease in MBP expression in the cell body.

Mature oligodendroglia were also seen to express the enzyme glutamine synthetase (GS) both *in vivo* and *in vitro* (R. Reynolds, unpublished observations).

When maintained in serum-containing medium, the O-2A progenitors differentiated into type-2-like astroglia as has been demonstrated in cells derived from optic nerve (Raff, 1989) and cerebellum (Levi *et al.* 1987; Curtis *et al.* 1988). After approximately a week in secondary culture, some type-2-like astroglia no longer expressed G_{D3}. The time period for which cells express these antigens may be elongated in culture compared to the *in vivo* situation and this may be why we fail to detect cells with this antigenic phenotype in cryostat sections: cells may cease to express G_{D3} before they begin to express GFAP *in vivo*. However, the reason such cells are not apparent in our sections may be due to the different nature of the antigens involved. Immunoelectron microscopical techniques would be needed to confirm our findings.

In conclusion, whilst the overall pattern of proliferation and differentiation of the preprogenitors, O-2A progenitors and oligodendroglia remained concordant in both our *in vivo* and *in vitro* studies, we have shown that the timing of antigen expression is often altered in culture. This appears to be the case for various stages along the developmental pathway, most notably for transitions between one antigenic profile and the next. Hence results obtained from *in vitro* data must only be extrapolated to the *in vivo* situation with caution.

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