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Oligodendrocyte progenitors isolated directly from developing telencephalon at a specific phenotypic stage: myelinogenic potential in a defined environment

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Summary

Oligodendroglia differentiate asynchronously in the developing central nervous system, passing through a series of stages identified by the sequential expression of specific differentiation antigens, culminating in the formation of the myelin sheath. In the work presented here, oligodendrocyte progenitors at a temporally narrow and well-defined phenotypic stage of development have been isolated in high purity and yield directly from postnatal rat telencephalon. This stage is identified by the expression of the O4 antigen, the earliest recognized surface marker specific for the oligodendroglial lineage, but the absence of the differentiation marker galactosylcerebroside (GalC). These $O4^+GalC^-$ progenitors first appear at birth (10^5/telencephalon), 2–3 days before $O4^+GalC^+$ oligodendrocytes. The work presented here demonstrates that a major subpopulation of $O4^+GalC^-$ progenitors (80 %), which we have termed 'proligodendrocytes', is fully committed to terminal oligodendrocyte differentiation. A relatively small, maximal set of nutritional supplements are sufficient for proligodendrocytes to carry out the myelinogenic cascade of differentiated gene expression in a temporally normal manner, in quantitatively significant amounts, in normal ratios of myelin protein isoforms, and in a regulated relationship to the inclusion of myelin-specific products into myelin-like membrane sheets. An important corollary is that this step of myelinogenesis does not require contact with other cell types, in particular neurones and astrocytes, nor does it require unknown growth factors unique to these cell types. Additionally under these conditions, there exists a developmentally quiescent subpopulation (20 %) of $O4^+GalC^-$ cells that may have significance for understanding the progenitors previously described in adult brain and suggested to be instrumental in remyelination under pathological conditions.

Key words: oligodendrocyte, progenitor, O4 monoclonal antibody, immunoselection, primary culture, myelinogenic potential, galactocerebroside, 2′,3′-cyclic nucleotide 3′-phosphohydrolase, myelin basic protein, proteolipid protein.

Introduction

During myelinogenesis in the central nervous system (CNS), oligodendroglia (OL) progress through a sequence of progenitor recruitment, proliferation, migration, and terminal differentiation steps. Although the overall process has been extensively characterized from a morphological perspective (Wood & Bunge, 1984), underlying mechanisms responsible for controlling the timing of OL differentiation, including the expression of specific myelin gene products, remain unclear. Myelinogenesis proceeds along a caudorostral gradient in the CNS, occurring at different times and rates among, and within, fiber tracts and cortical areas (Jacobsen, 1963; Skoff *et al.* 1976). This feature is typically reproduced in cultures initiated from CNS regions, as indicated by the asynchronous development of the arising OL population (reviewed in Pfeiffer, 1984).

Evidence for the developmental regulation of OL differentiation by other neural cell types remains controversial. Unlike Schwann cells of the peripheral nervous system, OL cultured in the absence of neurones can express the major glycolipids and proteins of the myelin sheath (Lemke, 1988). These include galactocerebroside (GalC; Mirsky *et al.* 1980), its sulfated ester sulfatide (Singh & Pfeiffer, 1985), 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP; McMorris, 1983; Bansal & Pfeiffer, 1985), myelin basic protein (MBP; Mirsky *et al.* 1980; Barbarese & Pfeiffer, 1981; Zeller *et al.* 1985), proteolipid protein (PLP; Macklin *et al.* 1986; Dubois-Dalcq *et al.* 1986), and myelin-associated glycoprotein (Dubois-Dalcq *et al.* 1986). Although the timing of myelin-specific gene expression can be

highly preserved in dissociated cultures (Abney *et al.* 1981; Zeller *et al.* 1985), plasticity in gene regulation has been observed. Inhibition or down-regulation of GalC expression (Keilhauer *et al.* 1985; Saneto & DeVellis, 1985) and delayed accumulation of MBP (Bologa-Sandru *et al.* 1981; Barbarese & Pfeiffer, 1981) implicate epigenetic factors, possibly of neuronal origin (Bologa *et al.* 1986). The interaction of premyelinating OL with an extracellular matrix component (Cardwell & Rome, 1988) and the stimulation of myelinogenesis by monoclonal antibody (mab) O4 (Bansal *et al.* 1988) suggest other modes of extrinsic regulation.

Three successive stages of OL development are distinguishable in the rat on the basis of the sequential expression of cell surface antigenic markers. The earliest is represented by the bipotential O-2A glial progenitor cell, first identified in cultures of postnatal rat optic nerve (Raff *et al.* 1983) by the appearance of gangliosides recognized by mab A2B5 (Fredman *et al.* 1984) and the expression of vimentin intermediate filaments (Raff *et al.* 1984). O-2A progenitors differentiate into either OL or type 2 astrocytes, with the latter process dependant on a specific inducing protein (Hughes *et al.* 1988). It is postulated that OL differentiation is timed by an intrinsic clock that counts cell divisions (Temple & Raff, 1986). They differentiate prematurely in defined medium to express GalC unless provided with platelet-derived growth factor produced by type 1 astrocytes (Richardson *et al.* 1988; Raff *et al.* 1988; Noble *et al.* 1988). O-2A cells correspond to bipotential cells in cerebellar cultures expressing the G_{D3} ganglioside (Levi *et al.* 1986, 1987). Immunohistochemical studies of the OL lineage in the developing cerebellum demonstrate that the G_{D3}+ phenotype identifies progenitors in the subventricular neuroectoderm (Goldman *et al.* 1984) and during their migration from subventricular germinal zones (Curtis *et al.* 1988; Reynolds & Wilkins, 1988).

A second stage in the OL lineage is identified by the appearance of the O4 surface antigen (Sommer & Schachner, 1981, 1982; Schachner *et al.* 1981). The $O4^+$ progenitor defines a transitional intermediate between the early bipotential O-2A progenitor stage ($A2B5^+/G_{D3}+/GalC^-$) and the onset of GalC expression (Sommer & Noble, 1986; Dubois-Dalcq, 1987; Levi *et al.* 1987). O4 is the earliest surface marker restricted to the OL lineage, distinguishing it from A2B5 and G_{D3} which are also expressed on other neuroectodermally derived cells (Goldman *et al.* 1984; Schnitzer & Schachner, 1982). Limited evidence suggests that $O4^+GalC^-$ cells no longer respond to an astroglial supplied mitogen (Keilhauer *et al.* 1985; Sommer & Noble, 1986) but may retain at least some glial bipotentiality (Sommer & Noble, 1986; Levi *et al.* 1987). Insulin, neurones, or other factors may be required for further differentiation into $GalC^+$ OL (Keilhauer *et al.* 1985; Dubois-Dalcq, 1987). A third stage of development is achieved when $O4^+$ progenitors express GalC, the prototypical surface marker for differentiated postmitotic OL (Raff *et al.* 1978), and rapidly lose specific surface gangliosides and intermediate filaments (Raff *et al.* 1984) before synthesizing myelin proteins.

Here we have approached the problem of the regulation of OL progenitor differentiation by determining the myelinogenic potential of a representative population of $O4^+GalC^-$ progenitors isolated directly from their normal germinal environment in the postnatal rat telencephalon. We have used solid-phase immunoselection in polystyrene Petri dishes to isolate $O4^+GalC^-$ progenitors in yields that permit biochemical analysis. We find that the majority of surviving, isolated progenitors differentiate in the absence of neurones and other macroglia in a simplified, hormone-supplemented culture medium, evolving a complex morphology and expressing the major components of myelin on a schedule characteristic of early myelinogenesis *in vivo*. On the other hand, a smaller population of these cells fails to express terminal myelinogenic markers. A preliminary report of this work has been made (Gard & Pfeiffer, 1988).

Materials and methods

Antibodies

Mouse hybridoma cell lines producing the 'O4' mab (Sommer & Schachner, 1981; Schachner *et al.* 1981); which recognizes sulfolipids (Singh & Pfeiffer, 1985; Bansal *et al.* manuscript in preparation), and two mabs for GalC, an IgM ('O1', Sommer & Schachner, 1981; Schachner *et al.* 1981; Singh & Pfeiffer, 1985) and an IgG (Ranscht *et al.* 1982), were provided by M. Schachner and M. Noble, respectively. The O1 and O4 mabs were fractionated from hybridoma culture medium consisting of serum-free medium supplemented with Nutridoma (Boehringer Mannheim) plus 1% fetal calf serum (Gibco) as described elsewhere (Bansal *et al.* 1988), snap frozen in liquid nitrogen and stored at −70°C. The final immunoglobulin content was quantified by dot–immunoblot assay and scanning densitometry using mouse myeloma Ig standards.

Other markers included mouse monoclonal anti-2′,3′-cyclic 3′-phosphohydrolase (CNP; Sprinkle *et al.* 1987; gift of T. Sprinkle) and rabbit polyclonal antisera specific for CNP (Bansal & Pfeiffer, 1985), myelin basic protein (MBP; Barbarese & Pfeiffer, 1981); and proteolipid protein (PLP; Macklin *et al.* 1982). Rat mabs (IgG) recognizing rat glial fibrillary acidic protein (GFAP; Lee *et al.* 1984*a*) and the $70\times10^3 M_r$ neurofilament subunit polypeptide (Lee *et al.* 1984*b*) were used as the unfractionated hybridoma culture medium (gifts of V. Lee). Mouse mabs (IgG) against vimentin and bromodeoxyuridine were purchased from Boehringer Mannheim and Becton-Dickinson, respectively. The A2B5 mab was concentrated 15-fold from hybridoma culture medium (clone 105, American Type Culture Collection CRL 1520) and biotinylated as described elsewhere (Goding, 1986).

Brain dissociation

Timed pregnant Sprague–Dawley rats (Charles River Breeding Laboratories) were used. The birthdate of litters was designated day 0 and was accurate to within 6 h of delivery. Individual telencephala from fetal, newborn and postnatal rat pups were dissociated into single cells as detailed elsewhere (Gard *et al.* 1988). Briefly, minced tissue was digested by mild trypsinization, mechanically dissociated by repeated pipeting against siliconized glass in the presence of DNAse and finally centrifuged through an underlayer of 4% (w/v) bovine serum

albumin to remove debris. A medium base of Hepes-buffered (20 mM, pH 7·4) Earle's balanced salt solution (EBSS–Hepes, 300–320 mosM), supplemented with 0·3 % (w/v) BSA (Fraction V; A-9647, Sigma) and 14 mM-glucose, was used throughout the procedure. Cell yields were determined by hemacytometry and viability by acridine orange/ethidium bromide uptake.

Isolation of $O4^+GalC^-$ progenitors

For the isolation of OL progenitors, the mechanical step of the dissociation technique (Gard *et al.* 1988) was modified. Pooled telencephala of six littermates, ages P4–P5, provided optimal yields. Following the trypsin digestion and its termination with trypsin inhibitor, the tissue mince was distributed evenly among four siliconized 50 ml conical glass centrifuge tubes and centrifuged for 5 min at 100 *g*. Pellets were resuspended to 5 ml/tube with EBSS–Hepes containing 80 $\mu g\,ml^{-1}$ DNAse (Sigma D-5025), 3 mM-$MgSO_4$ and 0·6 $\mu g\,ml^{-1}$ of the IgM, mab O1 (anti-GalC); the entire volume was forcefully pipeted 15 times against the side of the tube; and the tubes were then placed on ice for 15 min. The cells were centrifuged (above), the supernatants decanted, and the pellets resuspended to 2·5 ml with EBSS–Hepes containing 0·5 ml guinea pig complement (Cappel). The cells were pipeted another 5 times (above), left at room temperature for 45 min, and then centrifuged (above). After decanting the supernatants, pellets resuspended in 10 ml/tube EBSS–Hepes were left undisturbed for 5 min to allow settling of undissociated tissue fragments. The overlying single cell suspensions were transferred to separate tubes and centrifuged through 4 % BSA to remove debris (Gard *et al.* 1988).

The cell suspension of six telencephala ($5–7\times10^7$ cells) was resuspended in 48 ml EBSS–Hepes and divided among four 15×100 mm polystyrene bacteriological grade Petri dishes (Fisher Cat. No. 8-757-13) to remove fibroblasts and macrophages by differential adherence (20 min, 37 °C). Unattached cells, combined with loosely adherent cells removed by a single rinse of the dishes with EBSS–Hepes, were centrifuged for 5 min at 100 *g*. All remaining steps were carried out at 4 °C unless noted.

The pelleted cells were resuspended and labeled for 20 min with 3 $\mu g\,ml^{-1}$ of the O4 mab (freshly thawed sample) in 4 ml of EBSS–Hepes containing 5 % fetal calf serum (FCS) and 5 % normal goat serum (NGS) (both heat-inactivated; Gibco), then pelleted (above), washed once with 10 ml of EBSS–Hepes + 5 % FCS, and resuspended to 48 ml with EBSS–Hepes + 5 % FCS.

$O4^+$ cells have previously been purified by immunoaffinity selection using magnetic beads (Meier & Schachner, 1982). Here, immunolabeled $O4^+$ progenitors were selected from the total cell suspension by indirect binding to polystyrene bacteriological grade Petri dishes (above) that were precoated with anti-mouse IgM, using a procedure originally described for lymphocyte immunoselection (Wysocki & Sato, 1978), with modifications. Four dishes were each precoated for 3 h at 37 °C with 6 ml of 20 mM-phosphate buffer, pH 8·0, containing 8 $\mu g\,ml^{-1}$ affinity-purified goat anti-mouse IgM (Cappel) (conditions for saturable Ig adsorption to polystyrene were determined previously by solid-phase enzyme-linked immunoassay). After aspirating the unbound Ig (stored at 4 °C, the solution could be reused once), the dishes were rinsed twice with 15 ml of 10 mM-phosphate-buffered saline (PBS) and incubated with EBSS–Hepes + 5 % FCS for 1 h at 37 °C to block nonspecific cell attachment sites. The dishes were then each filled with 12 ml of the O4-labeled cell suspension (above) and placed on a level surface for 1 h. The contents were gently swirled after 30 min to redistribute unattached cells but otherwise left undisturbed. Unbound cells were removed using gentle suction and five successive rinses with 10 ml of DMEM + 1 % FCS. Bound cells were removed from the panning surface by mechanical shearing with ~3 ml EBSS–Hepes + 5 % FCS passed forcefully through flame-narrowed Pasteur pipets and centrifuged (as above) in 12×75 mm polystyrene tubes (Falcon). Yield and viability were determined as described above.

Cell culture

Cells were seeded on either poly-DL-ornithine-coated ($13–15\times10^3$ M_r, Sigma; 50 $\mu g\,ml^{-1}$, 1 h, 37 °C) glass coverslips (12 mm) within multiwells (Falcon 3047) in 20 μl droplets (10^4 cells) or in similarly treated microtiter wells (Falcon 3072) at various densities. Defined N2 medium (Bottenstein & Sato, 1979) was modified to contain those ingredients described by Eccleston & Silberberg (1984) that promote oligodendroglial development. These included D-glucose (4·5 $g\,l^{-1}$), human transferrin (50 $\mu g\,ml^{-1}$), bovine pancreatic insulin (5 $\mu g\,ml^{-1}$, Grade B, Calbiochem), 3,3,′5-triiodo-L-thyronine (15 nM) and sodium selenium (30 nM) in Dulbecco-modified Eagle's medium (Gibco). Additional supplements included D-biotin (10 nM) (Bottenstein, 1986), hydrocortisone (10 nM) (Warringa *et al.* 1987), sodium pyruvate (0·11 $mg\,ml^{-1}$) (Saneto & DeVellis, 1985), penicillin–streptomycin (100 i.u. and 100 $\mu g\,ml^{-1}$, respectively) and either 0·1 % BSA or 1 % heat-inactivated horse serum (Gibco) as sources of exogenous protein. Defined ingredients were obtained from Sigma unless noted. Medium was stored at 4 °C and made freshly every 2 weeks using frozen stock solutions, with the exceptions of insulin, transferrin and sodium pyruvate, which were freshly prepared.

To prepare conditioned medium, meninges were dissected from P4–P5 rat telencephala and cultured in DMEM containing 10 % FCS. After reaching confluency, the cells were passaged 1:4 and grown again to confluency, at which time the cultures contained <3 % $GFAP^+$ astrocytes (G. Gonye). The cultures were rinsed twice with DMEM and switched to modified N2 medium (above, without horse serum) for four days.

Immunofluorescence

Antibodies were diluted in EBSS–Hepes containing 5 % NGS and incubated with cells for 20 min at 4 °C unless noted. Unfixed cell suspensions (100 μl volumes) of dissociated telencephala and isolated progenitors were doubly labeled with a mixture of monoclonal anti-GalC (IgG; 1:40) and O4 (IgM; 1:100), and then with a mixture of TRITC–goat anti-mouse IgG (γ chain-specific; Southern Biotechnology) and FITC–goat anti-mouse IgM (μ chain-specific) $F(ab')_2$ fragments (Cappel), both diluted 1:50. After each labeling step, the cells were centrifuged in 3–4 ml of diluent. Fixation with 4 % *p*-formaldehyde in PBS was carried out during centrifugation (100 *g*, 10 min, 4 °C). The cells were washed once in PBS, attached to glass coverslips precoated with polyornithine and inverted on a droplet of PBS containing 50 % (v/v) glycerol (pH 8·6) and 2·5 % (w/v) diazobicyclo-(2,2,2) octane to suppress fading of fluorescence (Johnson *et al.* 1982). Cultures on coverslips were similarly labeled, fixed (30 min, 4 °C) and washed between steps with two 3–5 min changes of diluent. Live cultures labeled with either O4 or anti-GalC (IgM) and a TRITC conjugate (Cappel), as described above, were permeabilized with saponin (0·03 % in PBS, 10 min) after fixation, incubated with dilute NGS (5 % in PBS, 15 min) followed with either anti-CNP (either 1:100 polyclonal antiserum or undiluted hybridoma supernatant), anti-MBP (1:100), anti-PLP (1:40), anti-GFAP (1:10), anti-neurofila-

Table 1. *Postnatal expansion of $O4^+GalC^-$ progenitor and $O4^+GalC^+$ oligodendrocyte pools in the dissociated rat telencephalon*

Age	a Total $O4^+$ cells ($\times 10^5$)	b % $O4^+$ cells expressing GalC	c $O4^+GalC^-$ cells ($\times 10^5$)	d $O4^+GalC^+$ cells ($\times 10^5$)
E20	0·0 ± 0·0	0·0 ± 0·0	0·0	0·0
P0	1·1 ± 0·6	0·0 ± 0·0	1·1	0·0
P3	10·9 ± 1·6	7·8 ± 3·3	10·1	0·9
P4	19·4 ± 2·2	16·0 ± 3·9	16·3	3·1
P5	17·8 ± 4·1	18·1 ± 3·4	14·6	3·2

[a,b] Data (mean ± S.D.) are expressed per telencephalon ($n = 3$), each obtained from a separate litter and dissociated individually. Determinations are extrapolated values based on hemacytometric and double immunofluorescence labeling analyses of live cells aliquoted in suspension (detailed in Materials and methods).
[c,d] Cells per dissociated telencephalon calculated as the product of columns **a** and **b** for each age.

ment (1:5), or anti-vimentin (1:5) for 30 min, and finally labeled with FITC–conjugated goat anti-rabbit or anti-mouse IgG (γ chain-specific) at 1:50 dilution (Cappel; Southern Biotechnology). Simultaneous visualization of O4 and A2B5 markers (both IgMs) was carried out with freshly isolated and cultured progenitors by labeling cells first with O4 (FITC, above) followed by biotinylated A2B5 mab (1:5, referenced under '*Antibodies*') and then avidin–TRITC (1:300, Vector Laboratories). Non-immune rabbit serum (1:100) and mouse myeloma IgG (FLOPC-23, 1:50, Miles Scientific) were used as negative controls. Nuclear counterstaining with 4′-6,6-diamidino-2-phenylindole (DAPI, Polysciences) is described elsewhere (Bansal & Pfeiffer, 1985).

Cell proliferation

Cells were treated with 5-bromo 2′-deoxyuridine (BrdUrd, 10 μM, Sigma) during their first 18–36 h in culture. Live cultures were then indirectly labeled (FITC) with O4 or anti-GalC (IgM), fixed with 4 % p-formaldehyde in PBS (15 min, 4 °C) and doubly labeled for incorporated nuclear BrdUrd with mouse monoclonal anti-BrdUrd, according to Yong *et al.* (1988), and TRITC-conjugated goat anti-mouse IgG (γ chain-specific; 1:50, 1 h).

Electrophoresis and immunoblotting

Isolated $O4^+$ $GalC^-$ progenitors seeded at 2×10^5 cells cm^{-2} in polyornithine-coated polystyrene multiwells (Falcon) were cultured for 9 days in 33 % meningial culture-conditioned, modified N2 medium (see *Cell culture*). Cultures were rinsed twice with PBS, scraped from the substratum, centrifuged for 2 min at 12 000 *g*, and sonicated for 30 s in PBS. Samples were solubilized immediately in dissociation buffer consisting of 3 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 10 % (v/v) glycerol for either 2 min at 60 °C (for PLP detection) or 5 min at 100 °C (CNP and MBP). Protein samples (15 μg/lane) were electrophoresed through minigels (0·75 mm, Bio-Rad Mini-Protean apparatus) according to Laemmli (1970) at 100 V through a 4 % stacking gel and at 200 V through either a 10 % (for CNP detection) or 15 % (MBP and PLP) running gel. Frozen samples of myelin prepared from adult rat brainstem (Norton & Poduslo, 1973) and prestained molecular weight markers ($12{\cdot}3–200\times10^3$ M_r; Bethesda Research Laboratories) were run in parallel lanes.

After electrophoresis, proteins were transferred to nitrocellulose (0·45 μm pore size, Bio-Rad) according to Towbin *et al.* (1979) for 40 min at 50 mA (Mini Transblot apparatus, Bio-Rad). The blots were rinsed in PBS, blocked for 1 h in PBS containing 10 % (w/v) NGS and 3 % (w/v) BSA and incubated overnight at 4 °C with the appropriate dilutions of primary antisera in blocking buffer without NGS. Blots were washed six times for 5 min each in PBS before incubation for 2 h at room temperature with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:1000, Bio-Rad). Following another washing sequence (above), the blots were briefly rinsed in carbonate buffer (0·1 M-$NaHCO_3$, 1 mM-$MgCl_2$, pH 9·8) and then developed for 5–10 min in the same solution containing 0·03 % (w/v) *p*-nitro blue tetrazolium chloride and 0·015 % (w/v) 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

Results

Appearance of $O4^+GalC^-$ progenitors in rat telencephalon

The kinetics of the expression of the O4 antigen and GalC on OL progenitor cells in the late embryonic (E) and early postnatal (P) rat telencephalon were determined by immunofluorescence analysis of freshly dissociated tissue (Table 1). Both markers are resistant to mild trypsin digestion, permitting their detection on freshly isolated cells (Sommer & Schachner, 1981). The number of total viable cells recovered by dissociation declined progressively with age, from $22{\cdot}3(\pm2{\cdot}0)\times10^6$ cells at E20 to $15{\cdot}3(\pm2{\cdot}0)\times10^6$ at P5. Conversely, both the size and relative proportion of the $O4^+$ subpopulation ($O4^+GalC^-$ plus $O4^+GalC^+$) increased significantly during this interval (Fig. 1 and Table 1). Expression of the O4 antigen preceded that of GalC on dissociated cells, in accordance with a similar study of postnatal cerebellum (Schachner *et al.* 1981). Labeling with O4 was first detected at P0, on ~0·5 % of the cells, corresponding to $\sim10^5$ $O4^+$ cells per telencephalon. The number of $O4^+$ cells increased 10-fold by P3, and reached a plateau of $\sim1{\cdot}5\times10^6$ by P4–P5 (analyses of older pups were precluded by extensive debris in the preparations). Differentiated $GalC^+$ oligodendrocytes were first observed at P3 on ~8 % of the $O4^+$ cells. By P5 this value had increased to only 20 %, indicating that within the telencephalon there remained a substantial pool of $O4^+GalC^-$ progenitors.

Recovery of the $O4^+GalC^-$ population

The O4 mab is expressed on both $GalC^+$ OL and on their immediate progenitors (Sommer & Schachner,

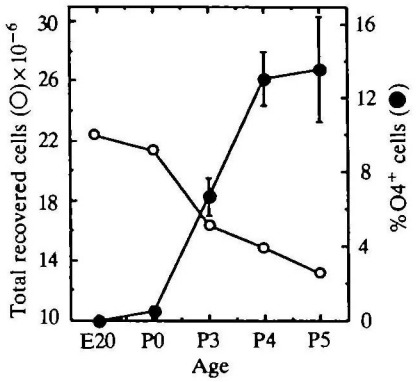

Fig. 1. Age-dependent inverse relationship between total cell recovery per dissociated telencephalon (○) and the proportion of cells expressing the O4 surface antigen (●). Data points represent the means of three independent experiments, each carried out with a single pup from a separate litter (see Table 1 legend for method of analysis).

1981, 1982; Schachner *et al.* 1981; Sommer & Noble, 1986; Dubois-Dalcq, 1987). Both cells types exist during the period of rapid progenitor development in the telencephalon (Table 1). Therefore, in order to study the developmental potential of only the $O4^+GalC^-$ population, complement-mediated immunolysis was first used to remove $GalC^+$ OL. The remaining $O4^+$ cells were >97% $GalC^-$, and were then isolated by solid-phase indirect immunoaffinity selection in polystyrene Petri dishes (Wysocki & Sato, 1978).

Total cell yields from six independent experiments averaged $3{\cdot}8(\pm 0{\cdot}5)\times 10^5$ cells isolated per telencephalon and consisted of 90 ± 1% $O4^+$ cells, <3% of which expressed GalC (Fig. 2). Therefore, yields of $\geq 2\times 10^6$ progenitors were routinely obtained from the pooled tissue of six animals in a single round of selection, representing a 20% recovery of the normal population found in freshly dissociated tissue. An additional 10% could be isolated by serially panning the unattached cell suspension in new dishes. Cell isolations carried out at postnatal ages beyond the P4–5 window resulted in a 50–70% reduction in yield.

Contaminant cells included (a) macrophages (5–6%), identified by the expression Fc receptors labeled with FITC–goat anti-rabbit IgG, (b) $A2B5^+04^-$ cells (2–3%) and (c) leptomeningeal cells (1–2%) containing vimentin intermediate filaments and expressing Thy-1 glycoprotein on their surfaces. The preparations were devoid of neurones, as judged by the absence of labeling with monoclonal anti-neurofilament antibody, and contained <0·1% $GFAP^+$ astrocytes, which survived poorly in the modified N2 medium.

Survival, growth, and proliferation of the $O4^+$ $GalC^-$ population in culture

Survival

Isolated progenitors were plated onto polyornithine-coated coverslips in a modified N2 defined medium (Materials and methods). More than 90% of the seeded cells attached to the substratum within 30 min. Although their initial viability exceeded 98%, pyknotic cells were observed by 12 h of culture. After 3 DIC the number of surviving $O4^+$ cells had decreased to ~25% and then remained relatively constant through 10 DIC before declining. Survival was partially dependent on the initial cell seeding density, increasing sharply at inputs ranging from $4\text{–}12\times 10^3$ cells/microculture. Survival after 3 DIC was not affected by increasing the concentration of horse serum to 10% or by substitution with 1% or 10% FCS. Seeding the cultures with as little as 1% FCS in the medium increased the proportion of $GFAP^+$ astrocytes at 3 DIC from essentially 0 to 5%; dual phenotypic $O4^+GFAP^+$ cells were only rarely observed. The presence of 10% FCS in the culture

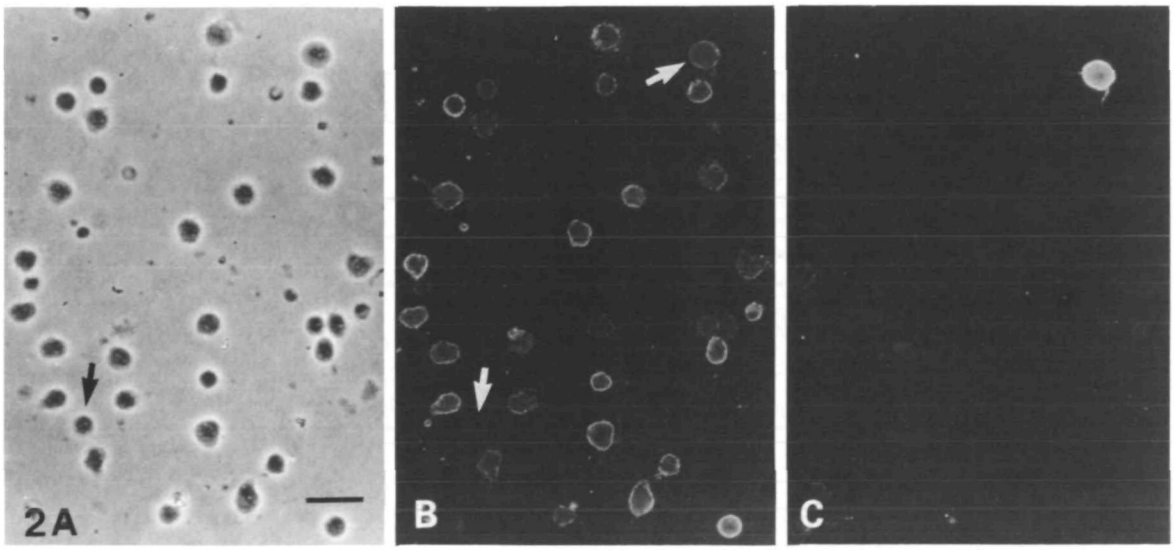

Fig. 2. Freshly isolated $O4^+GalC^-$ progenitors from P4 rat telencephalon under phase contrast (**A**), and doubly labeled by immunofluorescence for O4 (**B**) and GalC (**C**). A single, unlabeled contaminant cell (lower arrows, **A** and **B**) and a $GalC^+$ oligodendrocyte (upper arrow, **B**) are shown. Scale bar, 30 μm.

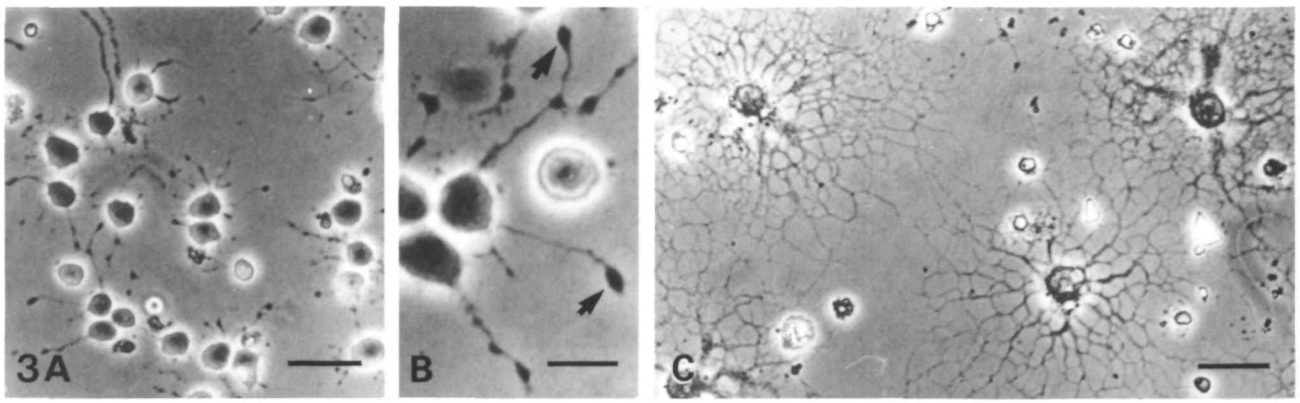

Fig. 3. Morphologies of live $O4^+GalC^-$ progenitors before and after their differentiation on a polyornithine substratum in modified N2 medium. A pattern of simple bipolar and multipolar process outgrowth typified most cells by 1 DIC (**A**). Terminal end bulb structures are indicated (arrows, **B**). At 7 DIC, biochemically differentiated OL displayed a complicated array of processes arranged in a symmetrical framework 150–200 μm in diameter. Scale bars, 40 μm (**A**,**C**); 15 μm (**B**).

medium produced a highly varied response among independent experiments in which initial process outgrowth during the first 24 h was either promoted or inhibited, before causing a substantial decrease in survival at 3 DIC to <5 %.

Morphology

The cell bodies of freshly isolated progenitors varied from 10–12 μm in diameter. After 1–2 DIC most cells had extended 2–5 delicate processes between 10 and 40 μm in length with terminal end bulbs and little apparent branching when viewed under phase-contrast optics (Fig. 3A,B). Immunofluorescence labeling with O4 revealed the presence of numerous filipodia and lamellipodia extending from these processes and limited secondary branching. By 3–4 DIC this simple, multipolar morphology had evolved to more complex forms, characterized by the profuse outgrowth of elongated processes and extensive secondary branching typical of OL in dissociated culture (Sommer & Schachner, 1981). Whereas the majority of the cells had processes uniformly distributed around the cell body, a smaller subpopulation had become arborized. By 7 DIC the processes of most cells had increased to 50–100 μm in length, becoming highly reticulate in their periphery (Fig. 3C) with expanded membranous sheets and leaflets (Fig. 4A,B) from which filipodia extended distally. This represented the final morphogenetic phase of progenitor development observed for the majority (~80 %) of cultured $O4^+$ cells during the 10-day course of study. Although nearly homogenous with respect to their phenotype at the time of isolation, not all progenitors evolved their morphology to the same extent or rate.

Proliferative capacity

Nuclear incorporation of bromodeoxyuridine (BrdUrd) during DNA synthesis was used as an index of progenitor proliferation and detected immunocytochemically. The labeling index of $O4^+$ cells doubly labeled with anti-BrdUrd (Fig. 5) remained constant at <2 % during the first 18–36 h in culture. During this period, many of the progenitors rapidly added GalC on their surface membrane (presented below). Fewer than 0·1 % of the immunolabeled $GalC^+$ OL were labeled with anti-BrdUrd after this interval, indicating that DNA synthesis was largely restricted to the $O4^+GalC^-$ progenitor population.

Developmental expression of the major myelin antigens

The differentiation potential of isolated proligodendrocytes was determined as a function of time in culture by double immunofluorescence labeling with O4 and a panel of antibodies recognizing GalC and the predominant myelin proteins CNP, PLP and MBP (Fig. 6).

$O4^+$ cells with little or no branching of their processes were consistently $GalC^-$. The expression of GalC, first on their cell bodies and then rapidly over the entire surface membrane, coincided with the appearance of more complex process outgrowth (Fig. 7A,B). Taken together, weak or intense labeling for GalC was observed on 40 % of the $O4^+$ cells by 2 DIC, >90 % by 4 DIC, and >95 % thereafter. Labeling with both markers was uniformly distributed across the surface of membrane sheets as they developed (Fig. 7C,D).

As O-2A bipotential glial progenitor cells ($A2B5^+GalC^-$) differentiate into $GalC^+$ oligodendrocytes in cultures of rat optic nerve, they cease to express A2B5 gangliosides and vimentin-containing intermediate filaments (Raff *et al.* 1984). Freshly isolated $O4^+GalC^-$ progenitors from telencephalon expressed A2B5-labeled gangliosides, which were rapidly lost upon differentiation into $GalC^+$ OL (R. Bansal, unpublished data). Whereas vimentin filaments were present in 60 % of the $O4^+GalC^-$ cells analyzed by immunofluorescence during the first 1–2 DIC, they were detected in only 4 % of newly differentiated $GalC^+$ (O1-labeled) OL in culture (Keilhauer *et al.* 1985).

The expression of CNP by differentiating progenitors followed a time course very similar to GalC. Examples of $GalC^-CNP^+$ cells were not found. The cell bodies of a small number of $GalC^+$ cells present among freshly

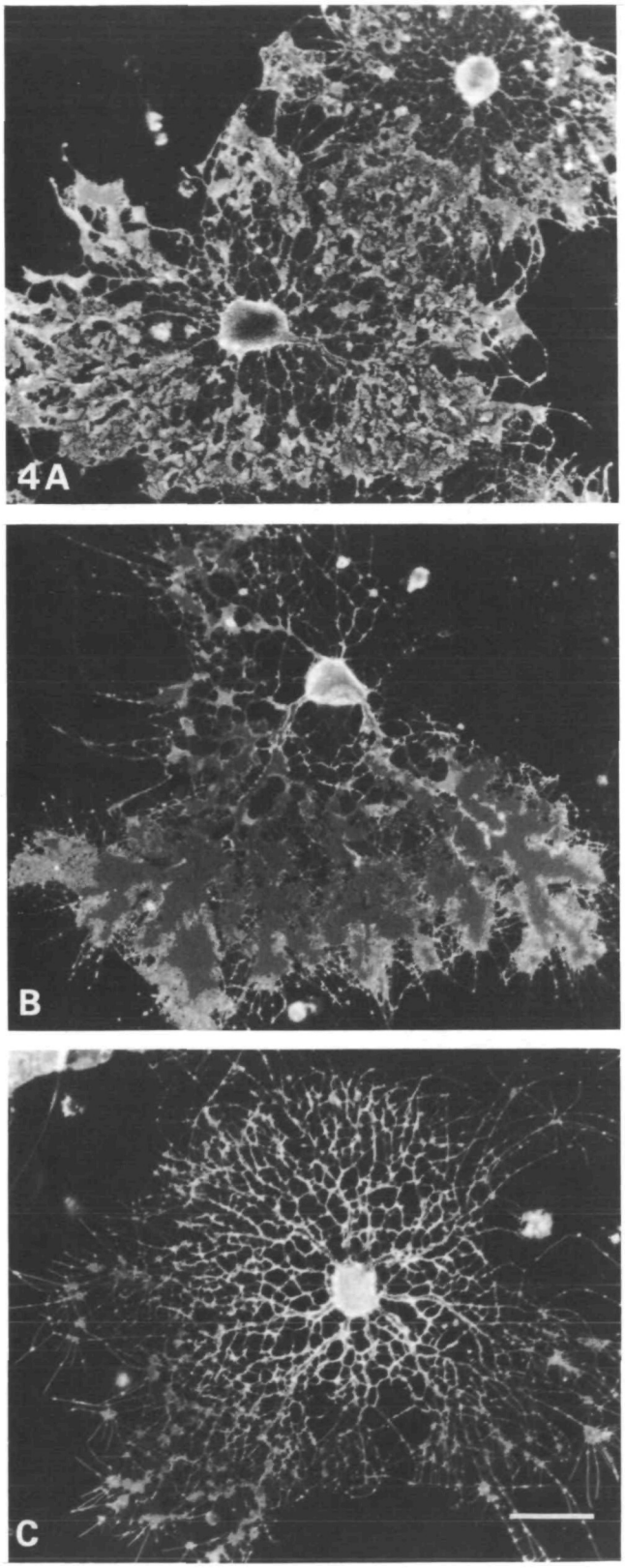

Fig. 4. Polymorphic OL labeled with O4 by immunofluorescence at 7 DIC. Most dispersed cells produced numerous leaflets of flattened membrane connected to a centrally positioned soma by radial processes (**A**). Examples of cells with a polarized membranous sheet (**B**), or with a highly reticulated network of processes with little membrane (**C**) also appeared, each possessing an outermost fringe of filipodia. Scale bar, 20 μm.

isolated progenitors were weakly labeled with monoclonal anti-CNP. Subsequently, >95% of the GalC$^+$ OL analyzed in culture contained CNP immunoreactivity, which intensified at 3–4 DIC concomittant with the development of a more complex process arrangement. Expanding membrane sheets were also brightly stained (Fig. 7F). The PLP appeared 2–3 days after GalC/CNP and abruptly in ~70% of O4$^+$ cells at 4 DIC. Immunoreactivity was restricted to the cell bodies of the first positive cells, but soon extended throughout the processes of many arborized cells (Fig. 8B). In more developed cells, PLP was concentrated within the cell body and large diameter processes, but was only faintly detectable in the surrounding membranous expansions (Fig. 8B). The onset and accumulation of MBP lagged 2–3 days behind PLP and 4–5 days behind GalC. MBP labeling was also initially restricted to the cell bodies of the first positive cells, appearing at the time of complex process branching (Fig. 9B), and then rapidly emerged in the central processes and expanded membranous sheets as development proceeded (Fig. 9E). Peripheral filipodia remained unlabeled (Fig. 9E). The proportion of differentiating progenitors expressing PLP and MBP reached a plateau value of ~80% at 7 and 10 DIC, respectively.

Bansal *et al.* (1988) observed a marked stimulatory effect of continuous O4 administration on OL differentiation in primary cultures of mixed cell type. To determine whether O4-immunolabeling during the progenitor isolation process may have affected myelin antigen expression in the present study, cell suspensions highly enriched in OL and their progenitors were mechanically sheared from primary glial cultures (McCarthy & DeVellis, 1980), seeded into culture and acutely labeled with O4 as described for immunoselection (20 min). The cells were allowed to differentiate and, when examined by immunofluorescence, showed no significant changes in the rate or extent of GalC or myelin protein expression in comparison to untreated cultures.

The synthesis of myelin polypeptides in differentiated progenitor cultures was analyzed by SDS–PAGE and immunoblotting. Cultures were maintained in meninges-conditioned medium, which significantly enhanced progenitor survival without appreciably altering the kinetic profile of myelin protein expression by immunofluorescence (work in progress). They were analyzed at 9 DIC and compared with a myelin standard prepared from adult rat brainstem. Under these conditions a small number of GFAP$^+$ astrocytes (6% of total cells) were present by 9 DIC.

Immunostained bands comigrating with CNP, PLP and MBP of myelin were observed in samples from differentiated progenitor cultures (Fig. 10). Of the two CNP polypeptides ($M_r = 46$ and 48×10^3) present in CNS myelin, only the predominant 46×10^3 form in rat

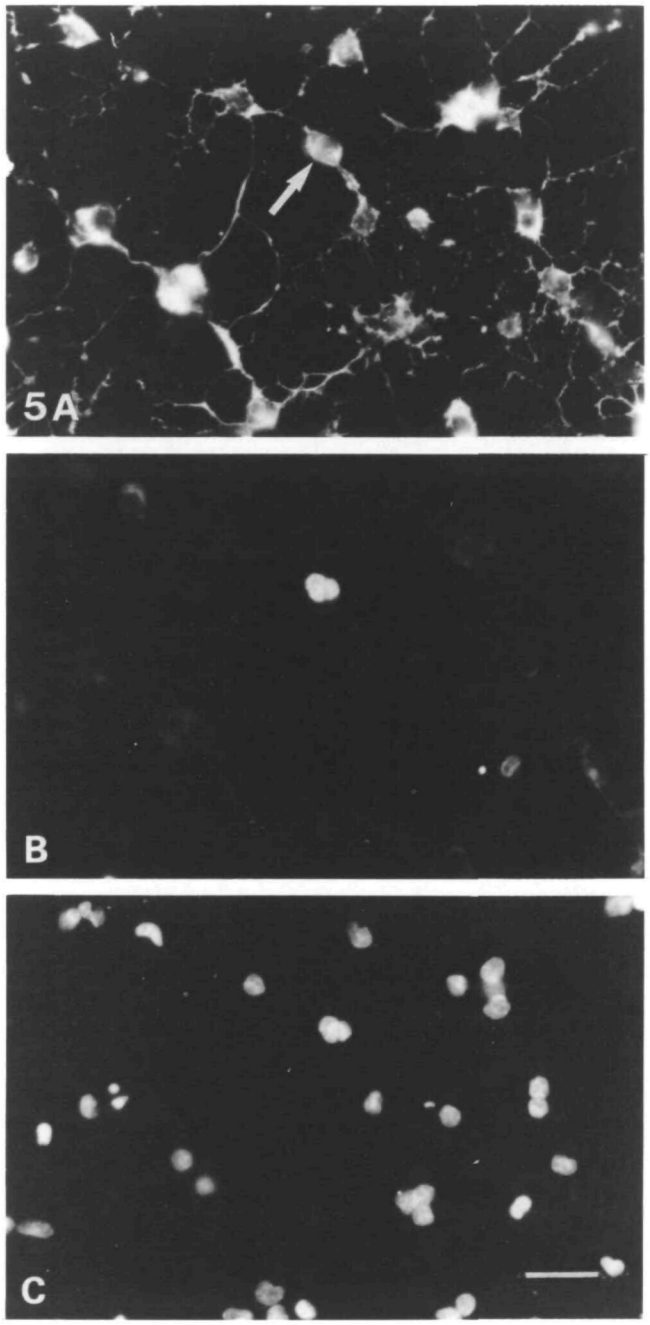

Fig. 5. $O4^+GalC^-$ progenitor proliferation *in vitro* visualized by BrdUrd immunocytochemistry. The nuclei of two partially overlapping $O4^+$ cells (**A**, arrow) were labeled with monoclonal anti-BrdUrd (**B**) after a 36 h exposure to the nucleoside analog in culture. All nuclei counterstained with DAPI (**C**). Scale bar, 40 μm.

(Bansal & Pfeiffer, 1985; Sprinkle *et al.* 1987) was readily detected in cultures. Anti-PLP serum labeled PLP ($M_r = 23\times10^3$) and the structurally related DM-20 proteolipid in rat myelin, as well as another unrelated myelin polypeptide ($M_r = 40\times10^3$; Macklin *et al.* 1982). Bands corresponding to PLP, a trace amount of DM-20 and a minor 27×10^3 M_r polypeptide not present in

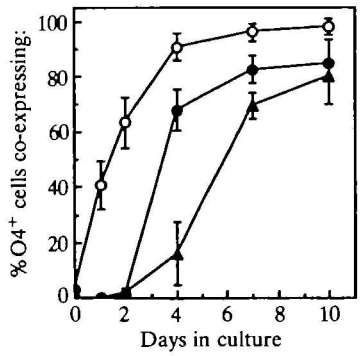

Fig. 6. Developmental time course of myelin-associated antigen expression by isolated progenitors *in vitro*. Each data point represents the mean percentage (±S.D.) of $O4^+$ cells doubly labeled with antibodies to GalC (○), PLP (●) or MBP (▲) in three independent experiments, each representing a separate litter, totaling 200–400 analyzed $O4^+$ cells.

myelin were detected in cultures. All four molecular weight forms of MBP ($M_r = 21{\cdot}5$, 18·5, 17 and 14×10^3) present in the rat CNS myelin (Barbarese *et al.* 1977) were synthesized in cultures initiated with isolated $O4^+GalC^-$ progenitors.

Developmentally quiescent subpopulation of $O4^+GalC^-$ progenitors

A smaller population (~15–20%) of $O4^+GalC^-$ cells failed to differentiate in culture during the 10 day course of study. GalC immunoreactivity became sparsely distributed on the plasma membrane surrounding the soma of many of these cells, but remained absent from their processes (Fig. 7D). They expressed neither the myelin proteins nor GFAP by immunofluorescence, and corresponded morphologically to cells at an earlier, undeveloped stage.

Discussion

We have analyzed the myelinogenic potential of progenitor cells reaching a critical stage in the OL lineage, identified by the $O4^+GalC^-$ surface phenotype. Here we have shown that the $O4^+GalC^-$ population which arises in the major germinal center of OL in the rat brain increases 15-fold during the first postnatal week, starting from ~10^5 cells at birth. This intermediate stage is predicted to last about 3 days *in vivo*, and the progenitor pool is of sufficient size to be isolated directly from the early postnatal rat telencephalon by cell immunoselection techniques. The yields are sufficient to minimize concerns regarding the extent to which the isolated cells represent the normal *in vivo* population, and provide material for many types of biochemical and molecular analyses. The cells under study are nearly homogenous ($O4^+GalC^-$) and, when introduced into culture, initially provide a simplified, synchronous starting population restricted to a narrow developmental window.

A maximal set of nutritional supplements and hor-

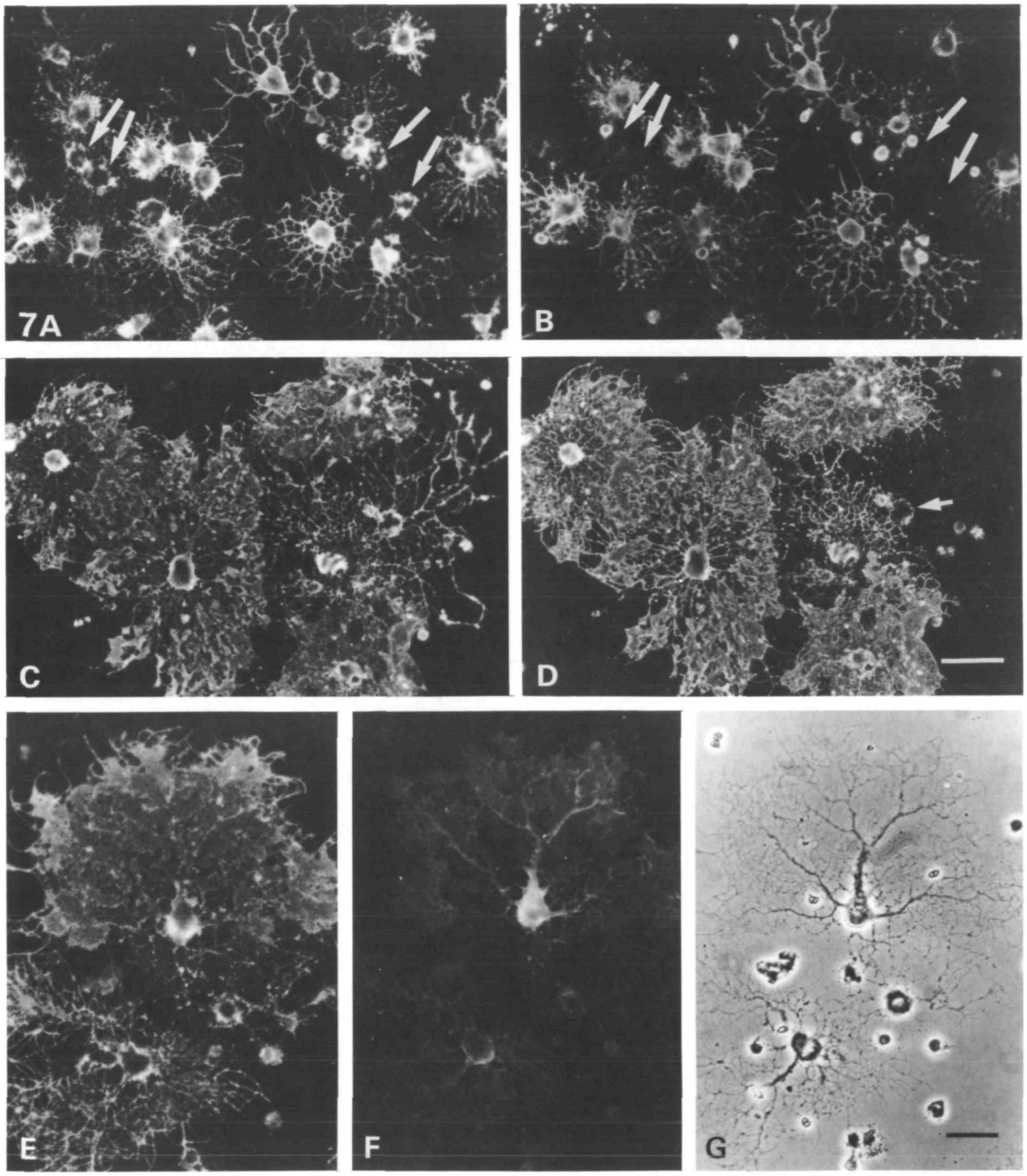

Fig. 7. Expression of GalC (**A–D**) and CNP (**E–G**) by isolated progenitors differentiating *in vitro*. The majority of $O4^+$ cells (**A**) after 2 DIC acquired varying amounts of GalC on their surfaces (**B**). Remaining $GalC^-$ progenitors (arrows, **A** and **B**) retained a simpler morphology. Whereas membrane-producing OL were uniformly labeled for O4 (**C**) and GalC (**D**) at 7 DIC, a single process-bearing cell in the field (arrow, **D**) expressed GalC only on its cell body. In a different field (**E–G**), three $O4^+$ cells (**E**) at 7 DIC exhibited faint, moderate, and intense CNP immunoreactivity (**F**), corresponding to cells with simple, intermediate and complex morphologies, respectively, under phase contrast (**G**). Scale bars, 40 μm (**A–D**); 30 μm (**E–G**).

mones are defined that permit the major subpopulation (80%) of isolated $O4^+GalC^-$ cells to carry out a sophisticated cascade of differentiated gene expression in a temporally normal manner, in quantitatively significant amounts, and in apparently normal ratios of myelin protein isoforms, while coordinately evolving a two-dimensional morphology consistent with their axon-ensheathing counterparts *in vivo* (Bunge, 1970). From these data, we conclude that a specific ('committed'), regulatory program of OL development is in

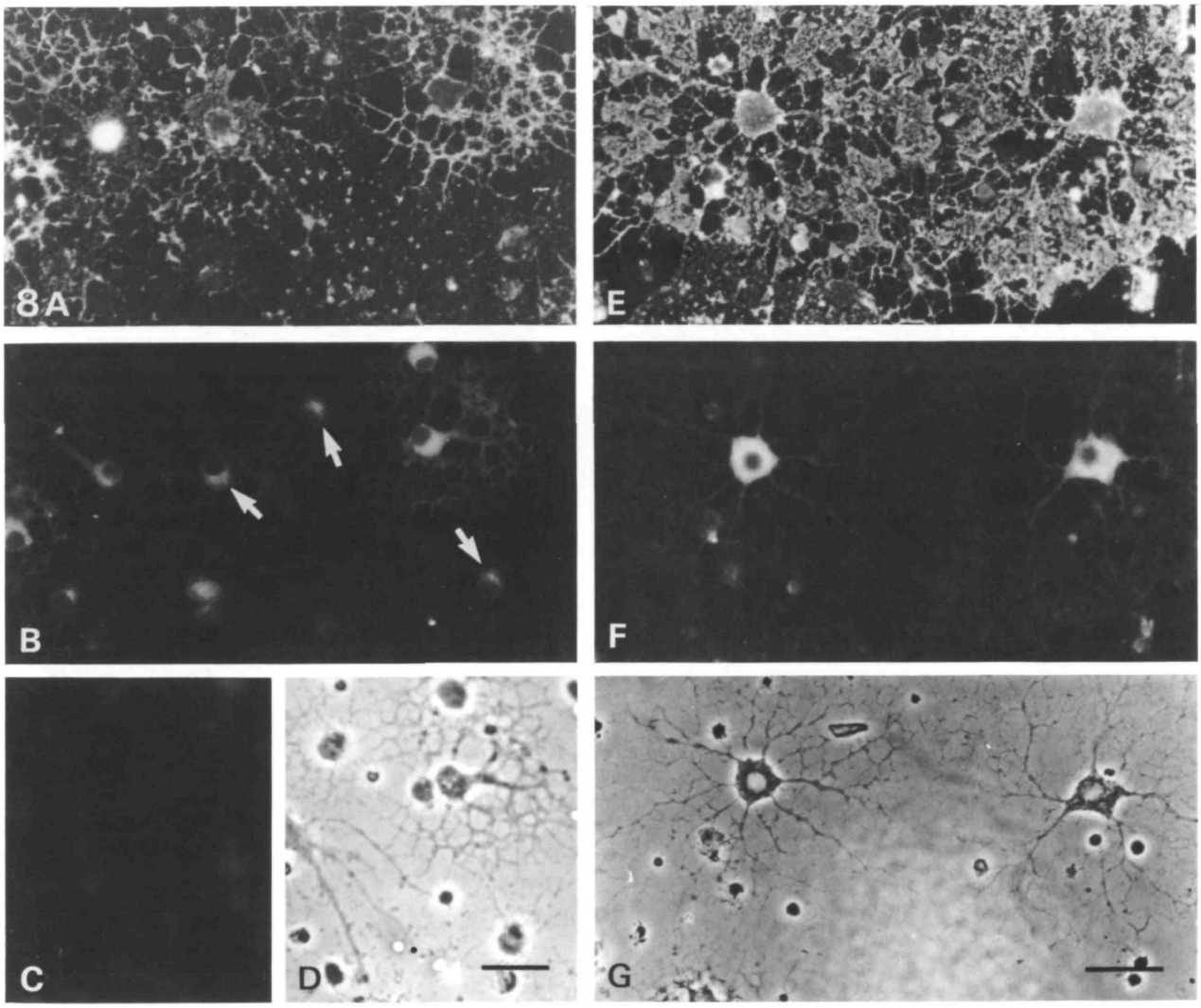

Fig. 8. Immunofluorescence labeling of differentiated proligodendrocytes expressing PLP in culture. At 4 DIC, most $O4^+$ cells (**A**) displaying complex process outgrowth contained PLP in the perinuclear cytoplasm (arrows, **B**); cells with more developed arborizations stained throughout their processes; (**C**) background labeling with nonimmune rabbit serum; (**D**) phase contrast corresponding to a portion of the field in **A**,**B**. At 7 DIC, $O4^+$ OL (**E**) show intensely bright PLP immunoreactivity restricted primarily to cell bodies and larger processes (**F**); (**G**) corresponding field under phase contrast. Scale bars, 40 μm (**A–D**); 30 μm (**E–G**).

place, in cells at this stage, which we term here 'proligodendrocytes.' Not all of the components in the medium are necessarily required. Some may serve roles other than the regulation of differentiation *per se*. Nevertheless, the immunohistochemical localization of transferrin and biotin to OL *in situ* (Connor & Fine, 1987; Levine & Macklin, 1988) supports a role for their growth-supporting effects in culture (Eccleston & Silberberg, 1984; Saneto & DeVellis, 1985; Bottenstein, 1986). Multiple parameters of myelinogenesis *in vitro* are stimulated by hydrocortisone (McCarthy & DeVellis, 1980; Saneto & DeVellis, 1985; Warringa *et al.* 1987), triiodothyronine (e.g. Eccleston & Silberberg, 1984; Almazan *et al.* 1985; Shanker *et al.* 1987) and insulin (Eccleston & Silberberg, 1984; Saneto & DeVellis, 1985; van der Pal *et al.* 1988), the latter attributed to interaction with receptors for insulin-like growth factor I (McMorris *et al.* 1986). Our findings do not neccessarily preclude a role for neurons and astrocytes in providing factors regulating OL differentiation (Bologa-Sandru *et al.* 1981; Barbarese & Pfeiffer, 1981; Bologa *et al.* 1986; Macklin *et al.* 1986). For example, insulin-like growth factor I may be supplied during myelinogenesis from neuronal or astrocytic sources (Rotwein *et al.* 1988).

Cultured proligodendrocytes differentiated on a schedule closely resembling early myelinogenic activity *in vivo*. The appearance of GalC after 1–3 DIC reproduced the normal time lag for the appearance of this marker observed among $O4^+$ cells freshly dissociated from the neonatal telencephalon. Similarly, the sequential expression in culture of CNP and MBP within <1 and 4–5 days, respectively, of GalC also closely mimics the highly conserved order and relative timing of

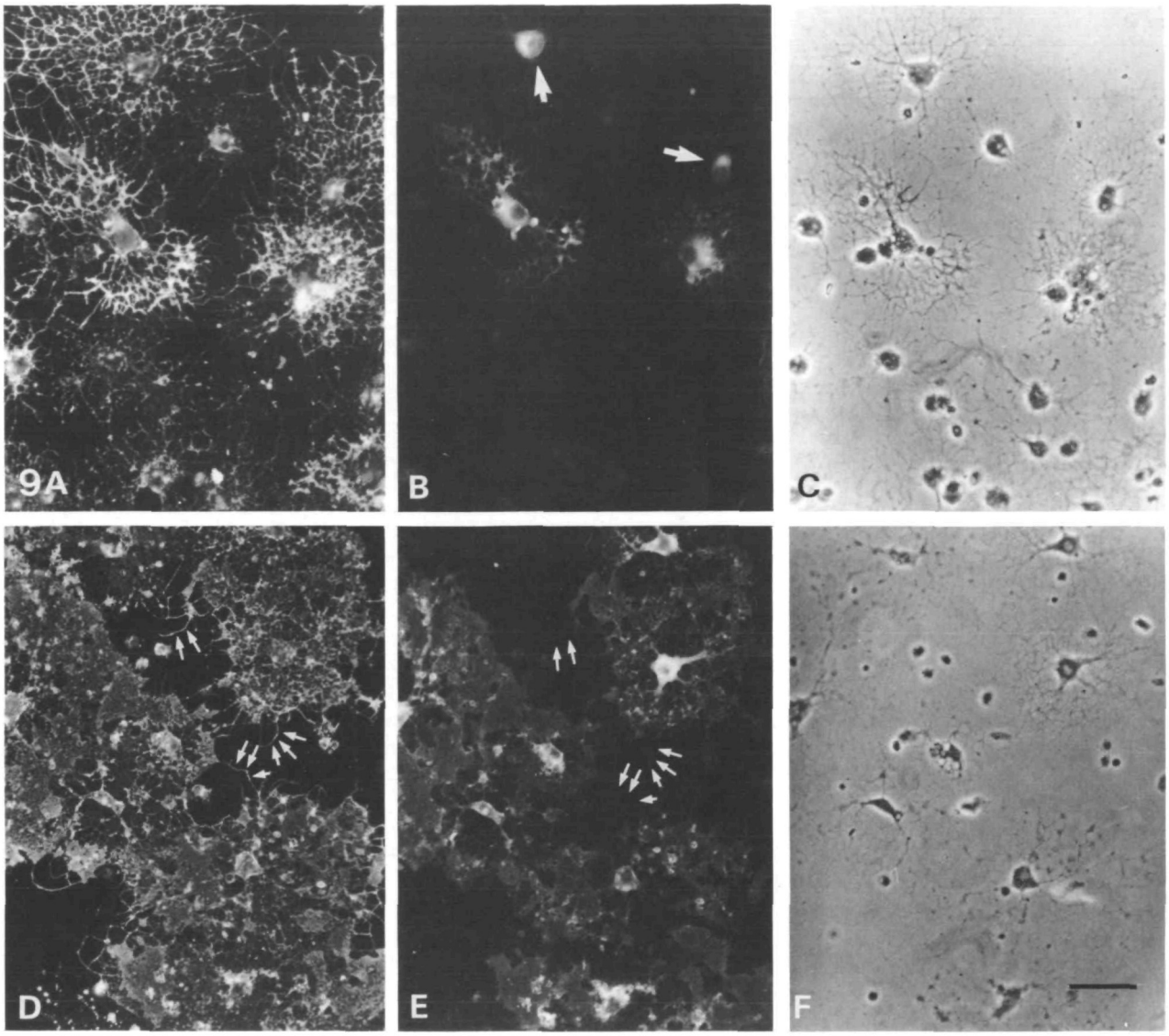

Fig. 9. Emergence of MBP immunoreactivity in differentiating $O4^+GalC^-$ progenitors after 4 DIC (**A–C**) and 7 DIC (**D–F**). A field of several $O4^+$ cells (**A**) includes several doubly labeled for MBP (**B**), expressed initially in the cell bodies (arrows) and processes. By 7 DIC, most MBP^+ OL are labeled throughout their cytoplasm, processes and membranes, but not in the peripheral filipodia (**E**, small arrows). The corresponding fields under phase contrast are in **C** and **F**. Scale bar, 40 μm.

antigen expression detected immunohistochemically among OL differentiating in regions of the developing rat brain (Reynolds & Wilkins, 1988). From another perspective, the emergence of CNP and MBP in cultured proligodendrocytes at ages corresponding to postnatal days 7–8 and 10–12, respectively, occurs on a schedule congruent with the onset of rapid accumulation of these markers in the rat cerebrum (Cohen & Guarnieri, 1976; Sprinkle *et al.* 1978; Bansal & Pfeiffer, 1985). Similarly, the emergence of PLP at 4 DIC, slightly ahead of MBP, is consistent with the observation of PLP immunoreactivity in newly myelinating cortical tracts by postnatal day 10 (Trapp *et al.* 1987), the temporal coexpression of PLP and MBP mRNAs *in situ* (Verity & Campagnoni, 1988) and ordered gene expression by differentiating OL progenitors generated *in vitro* (Macklin & Pfeiffer, 1983). Nevertheless, in another culture system the reverse order of MBP and PLP expression has been reported (Dubois-Dalcq *et al.* 1986).

Complex process outgrowth accompanied the sequential expression of myelin-associated antigens, and led 1–3 days later to the elaboration of membrane sheets. These changes in morphology parallel the morphogenesis of OL *in situ* through a 'lacy' stage, during which the synthesis of myelin proteins commences and appropriate axons are contacted, shortly before myelination begins (Sternberger *et al.* 1978; Friedrich & Sternberger, 1983). OL in dissociated cultures of mixed cell type produce biochemically differentiated, myelin-like membrane (Pfeiffer, 1984; Dubois-Dalcq *et al.* 1986; Knapp *et al.* 1987). Here, in the absence of neurones and astrocytes, a polycationic substratum alone served as a permissive template for substantial

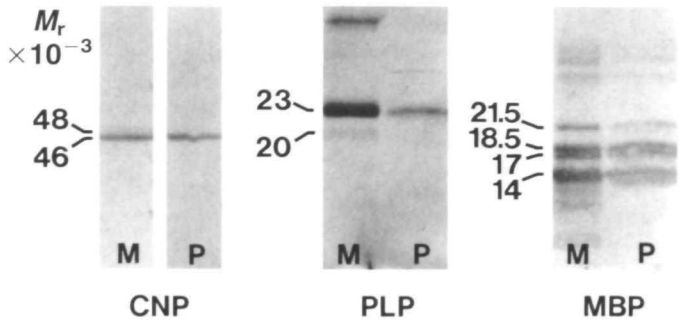

Fig. 10. Immunoblots of rat CNS myelin (**M**) and cultured progenitors (**P**) harvested after 9 DIC. Indirect labeling with rabbit polyclonal antisera against CNP (1:500), PLP (1:100) and MBP (1:1000) revealed polypeptide bands in culture samples corresponding to authentic CNP ($M_r = 46 \times 10^3$), PLP ($M_r = 23 \times 10^3$) and MBP ($M_r = 14$, 17, 18·5 and $21{\cdot}5 \times 10^3$) in myelin.

membrane sheet formation by differentiating proligodendrocytes. CNP immunoreactivity was uniformly distributed throughout these membranes, more similar to the adaxonal myelin wrappings which ensheath the axon, rather than unfurled compact myelin of which CNP is not a major component (Trapp *et al.* 1988). The formation of a relatively immature myelin-like membrane in these cultures may also explain why PLP, the predominant and final constituent protein inserted into compact myelin (Monge *et al.* 1986), was restricted largely to the site of its synthesis in the perinuclear cytoplasm (Trapp *et al.* 1987). Alternatively, the transport and assembly of PLP into the expanded membrane sheets may be blocked under the present circumstances, possibly requiring additional environmental information from the neurone itself (Macklin *et al.* 1986). Another distinctive characteristic of these membranes was the outgrowth of filipodia from their periphery. Similar structures emanating from the processes of $GalC^+$ OL at earlier morphological stages in culture have been proposed to function in bidirectional vesicular transport and intercellular recognition processes (Kachar *et al.* 1986). Their role in the ensheathment of appropriate axons by the newly assembled myelin membrane sheetlet can also be envisioned.

In addition to the major population of rapidly differentiating cells, a smaller subpopulation of $O4^+GalC^-$ cells (termed, with tongue in cheek, 'procrastocytes') failed to differentiate into morphologically or biochemically recognizeable OL. These cells expressed little or no detectable GalC on their surfaces and remained unlabeled with myelin protein-specific antisera throughout the course of study. It is unlikely that these cells arose in culture from contaminating $A2B5^+O4^-$ progenitors, which may have been blocked in their subsequent differentiation. Such cells were infrequent in the freshly isolated starting populations (2–3 %) and, in addition, were without significant levels of platelet-derived growth factor required for their proliferation (Noble *et al.* 1988; Raff *et al.* 1988; Richardson *et al.* 1988). It is interesting to speculate regarding the origin of a quiescent subpopulation unable to differentiate in culture. On the one hand, the activation of the cascade of terminal myelinogenic differentiation may occur at an intermediate point within the time span of the $O4^+GalC^-$ developmental stage; $O4^+$ cells removed from required stimulatory factors present *in vivo* (but absent in culture) prior to this critical regulatory event would be unable to progress further. This model predicts that the two subpopulations are merely sequential stages of a common lineage, and further subdivides the $O4^+$ compartment. Alternatively, there may be two phenotypically distinct $O4^+GalC^-$ subpopulations with different environmental requirements for further lineage progression. In this case, factors required for progression by the major, but not the minor, subpopulation must be present in the defined medium. Both possibilities are intriguing in light of the evolving hypothesis that the occurence of limited remyelination in the CNS under pathological conditions, such as multiple sclerosis, may involve factors regulating activation of a cryptic population of glial progenitors (Ludwin, 1989), which evidently persist into adulthood (ffrench-Constant & Raff, 1986).

In conclusion, we believe that the data support the concept that isolated $O4^+GalC^-$ cells demonstrate in culture the myelinogenic potential of an important developmental intermediate existing *in vivo* and constitute a highly simplified system in which to further probe the complex details of cellular differentiation and growth control.

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