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The *in vitro* differentiation of a bipotential glial progenitor cell

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We have studied the properties of a glial progenitor cell from 7-day-old rat optic nerve that differentiates *in vitro* into an oligodendrocyte if cultured in serum-free medium and into an astrocyte if cultured in foetal calf serum (FCS). Using galactocerebroside as a marker of oligodendrocyte differentiation and glial fibrillary acidic protein as a marker of astrocyte differentiation, we show that the acquisition of these marker molecules occurs rapidly in culture and requires both RNA and protein synthesis. We provide evidence that the effect of FCS on the development of the glial progenitor cell is not due to its influence on cell-substrate adherence or actin filament organization and is not mimicked by an increase in intracellular cyclic AMP, cyclic GMP or pH. The progenitor cell contains vimentin filaments and retains them on becoming an astrocyte but loses them on becoming an oligodendrocyte. Most importantly, we show that the choice of developmental pathway taken by the bipotential glial progenitor cells in culture is reversible for 1 – 2 days and then becomes fixed, at least under the conditions we studied.

Key words: astrocytes/differentiation/glial progenitor cell/oligodendrocytes

Introduction

The rat optic nerve contains three types of macroglial cells – oligodendrocytes, which make myelin in the central nervous system (CNS), and two kinds of astrocytes (type 1 and type 2) (Raff *et al.*, 1983a), whose functions are still uncertain. The three cell types can be distinguished by antibodies: anti-galactocerebroside (GC) antibodies react exclusively with the oligodendrocytes (Raff *et al.*, 1978), antibodies against glial fibrillary acidic protein (GFAP) (Bignami *et al.*, 1972) react with both types of astrocytes (Raff *et al.*, 1983a), while the A2B5 monoclonal antibody (Eisenbarth *et al.*, 1979) reacts with type 2 but not type 1 astrocytes (Raff *et al.*, 1983a; Miller and Raff, 1984). Thus the antigenic phenotypes of the three cells are: oligodendrocytes, GC^+,$GFAP^-$; type 1 astrocytes, $A2B5^-$,$GFAP^+$; and type 2 astrocytes, $A2B5^+$, $GFAP^+$. In the developing rat optic nerve, type 1 astrocytes first appear at embryonic day 16, oligodendrocytes at 2 days after birth and type 2 astrocytes during the second post-natal week (R.H.Miller, S.David, E.R.Abney and M.C.Raff, in preparation).

We have previously described an $A2B5^+$,$GFAP^-$,GC^- bipotential glial progenitor cell in 7-day-old rat optic nerve that develops into a type 2 astrocyte if cultured in the presence of foetal calf serum (FCS) and into an oligodendrocyte if cultured in serum-free medium (Raff *et al.*, 1983b). This is the first example of a normal progenitor cell in the mammalian CNS that can be induced to develop into either of two very different cell types depending on the culture conditions. Here we address the following questions concerning the *in vitro* differentiation of this oligodendrocyte-type 2 astrocyte (O-2A) progenitor cell. (1) How rapidly can it differentiate in culture? (2) Are RNA and protein synthesis required for differentiation? (3) Does FCS influence differentiation by affecting cell-substrate adherence and/or actin filament organization – factors that have been reported to affect dramatically the differentiation of some cell types *in vitro* (Archer *et al.*, 1982; Zanetti and Solursh, 1984; Spiegelman and Ginty, 1983)? (4) Can the effect of FCS be mimicked by increasing intracellular cyclic AMP, cyclic GMP or pH? (5) Does the O-2A progenitor have intermediate filaments and lose them on becoming an oligodendrocyte, or does it lack such filaments and gain them on becoming an astrocyte? (6) To what extent is the choice of differentiation pathway taken by an O-2A progenitor cell reversible?

Results

O-2A progenitor cells differentiate rapidly in culture

Less than 1% of the $A2B5^+$ cells in fresh suspensions of 7-day-old optic nerve were $GFAP^+$. After 1 day in culture in DMEM-FCS, ~40% of the $A2B5^+$ cells had acquired $GFAP^+$ filaments and this proportion increased to >90% by 2 days in culture (Table I).

To study the acquisition of GC by $A2B5^+$ O-2A progenitor cells, it was first necessary to kill the existing GC^+ oligodendrocytes (which constituted 5 – 10% of the cells in suspensions of 7 day optic nerve) by treating the cell suspensions with anti-GC antibody and complement. Less than 1 in 500 cells was GC^+ after this treatment (not shown). When such cell suspensions were cultured in serum-free DMEM, ~10% of the $A2B5^+$ cells were weakly GC^+ by 1 day, and this proportion increased to >50% by 2 days and >80% by 3 days in culture (Table I). As previously described (Raff *et al.*,

Table I. Timing of O-2A progenitor cell differentiation[a]

Time in culture (days)	Culture medium	Proportion (%) of $A2B5^+$ cells expressing GFAP	GC
1	DMEM-FCS	46 ± 9	2 ± 0.6
2	DMEM-FCS	91 ± 2	2 ± 0.5
3	DMEM-FCS	96 ± 1	1 ± 0.5
1	serum-free DMEM[b]	2 ± 2	11 ± 2
2	serum-free DMEM[b]	4 ± 2	67 ± 5
3	serum-free DMEM[b]	3 ± 1	83 ± 4

[a]Seven day optic nerve cells were grown on PLL-coated glass coverslips for 1, 2 or 3 days before being stained. Results in this and the following tables are expressed as mean ± S.D. of at least three separate experiments.
[b]In this and the following table, cells cultured in serum-free DMEM were treated with anti-GC antibody and complement prior to culture.

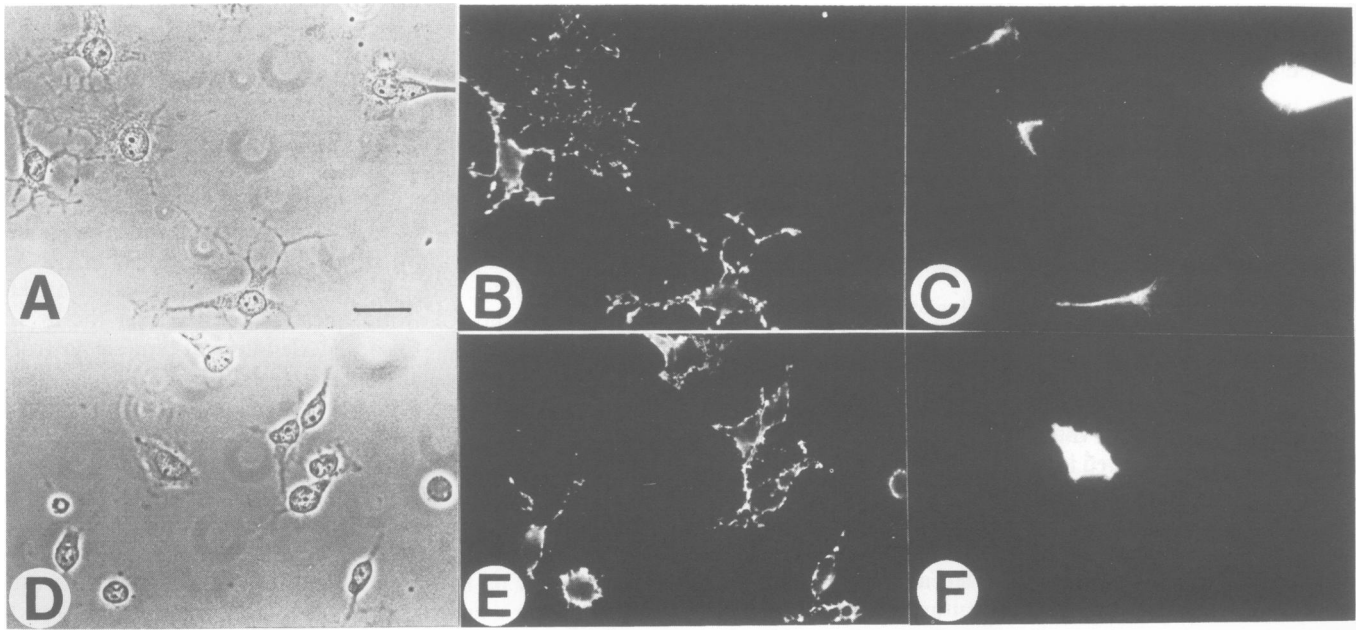

Fig. 1. Effect of actinomycin D on the induction of GFAP expression in A2B5$^+$ O-2A progenitor cells. Cultures of 7 day optic nerve cells were maintained in DMEM-FCS for 1 day before they were labelled with A2B5 antibody followed by G anti-MIg-Rd; after fixation with acid-alcohol, they were labelled with anti-GFAP serum followed by Sh anti-RIg-Fl and viewed with phase contrast (**A** and **D**), rhodamine (**B** and **E**) and fluorescein (**C** and **F**) optics. The cells in **D**, **E** and **F** were treated with actinomycin D (0.5 μg/ml) for 1 h soon after plating. Note that three out of four A2B5$^+$ cells have become GFAP$^+$ in the untreated culture while none of the A2B5$^+$ cells are GFAP$^+$ in the treated culture. One GFAP$^+$ type 1 astrocyte is seen in **C** and another in **F**. Scale bar = 20 μm in this and the following four figures.

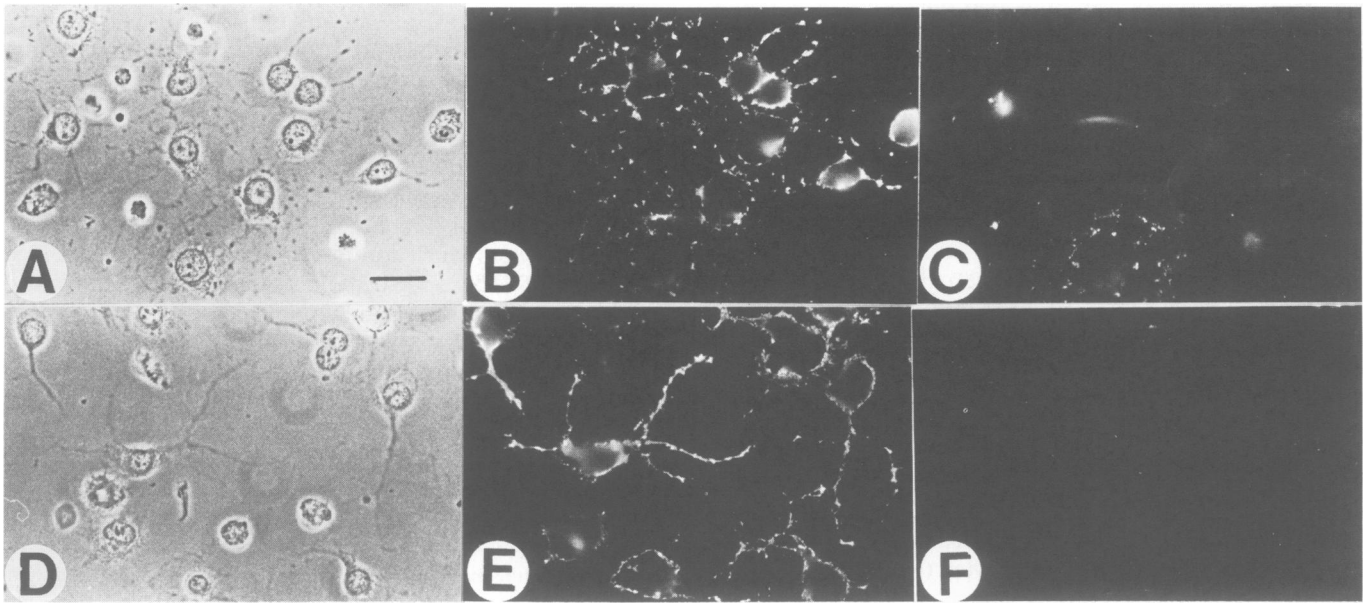

Fig. 2. Effect of cycloheximide on the acquisition of GC by A2B5$^+$ O-2A progenitor cells. Seven day optic nerve cells were treated with anti-GC and complement and then cultured in serum-free DMEM for 1 day before they were labelled with anti-GC antibody followed by anti-IgG3-Fl and then A2B5 antibody followed by anti-IgM-Rd. After fixation they were viewed as in Figure 1. The cells shown in **D**, **E** and **F** were treated with cycloheximide (5 μg/ml) for 4 h soon after plating. Note that several A2B5$^+$ cells have become weakly GC$^+$ in the untreated culture while none are GC$^+$ in the treated culture.

1983b), relatively few A2B5$^+$ cells became GC$^+$ in DMEM-FCS or GFAP$^+$ in serum-free DMEM (Table I).

Differentiation requires RNA and protein synthesis

When 7 day optic nerve cells were treated with actinomycin D (0.5 μg/ml) for 1 h to inhibit RNA synthesis or with cycloheximide (5 μg/ml) for 4 h to inhibit protein synthesis beginning 30 min after plating, A2B5$^+$ cells failed to acquire GFAP when cultured in DMEM-FCS (Figure 1) or GC when cultured in serum-free DMEM for 1 day (Figure 2, Table II). Most of the cells in such cultures looked healthy and excluded trypan blue, although the A2B5$^+$ cells extended fewer processes than they did in control cultures (Figures 1 and 2). Whereas almost all of the cells in actinomycin D-treated cultures died by 3 days, many survived in cycloheximide-treated cultures in DMEM-FCS and by 3 days, 50–70% of

the A2B5$^+$ cells were GFAP$^+$ (Table II); almost all of the cells died in cycloheximide-treated cultures in serum-free DMEM.

O-2A progenitor cells differentiate into astrocytes even on a relatively non-adherent surface or in the presence of cytochalasin D

To determine whether FCS induces type 2 astrocyte differentiation by increasing cell adherence to (and therefore spreading on) the culture dish, we cultured 7 day optic nerve cells on non-coated bacteriological dishes in DMEM-FCS. After 3 days in culture, >90% of the A2B5$^+$ cells had acquired GFAP to become type 2 astrocytes; although most of these cells had one or more processes, they spread relatively little and adhered only weakly to the culture dish compared with type 2 astrocytes growing on poly-L-lysine (PLL)-coated coverslips or tissue culture dishes (not shown).

When 7 day optic nerve cells were cultured on PLL-coated coverslips in DMEM-FCS in the presence of the actin-filament-disrupting drug cytochalasin D (2 μg/ml) for 3 days, >85% of the A2B5$^+$ cells acquired GFAP and were GC$^-$, although most of these cells contained less GFAP than did type 2 astrocytes in control cultures and the GFAP$^+$ filaments were often poorly organized. As expected, many of the cells in the cultures treated with cytochalasin D had unusual morphologies and/or were multinucleated. While some A2B5$^+$,GFAP$^+$ type 2 astrocytes were binucleate, others were mononucleate (Figure 3), indicating that they had not undergone nuclear division during the 3 days in culture.

The effects of FCS are not mimicked by increasing intracellular cyclic AMP, cyclic GMP or pH

To determine whether FCS induces O-2A progenitor cells to differentiate into type 2 astrocytes by increasing intracellular cyclic AMP, 7 day optic nerve cells were cultured in serum-free DMEM in the presence of dibutyryl cyclic AMP (10^{-3} M) or cholera toxin (200 ng/ml) for 3 days. Many oligodendrocytes but very few type 2 astrocytes developed in such cultures and the ratio of these two glial cell types was indistinguishable from that in control cultures (Table III). Similarly, neither dibutyryl cyclic GMP (10^{-3} M) nor NH_4Cl (10 mM) (which would be expected to increase intracellular pH (Hutchens *et al.*, 1939; Epel *et al.*, 1974) induced type 2 astrocyte development in culture (Table III). Moreover, none of these agents had a significant influence on the development of type 2 astrocytes in cultures grown in DMEM-FCS (not shown).

O-2A progenitor cells contain vimentin filaments

To determine whether O-2A progenitor cells contain vimentin intermediate filaments (IFs), freshly dissociated 7 day optic nerve cells were doubly labelled with A2B5 and anti-vimentin antibodies: 80–90% of the A2B5$^+$ cells were vimentin positive (not shown). Although most of the A2B5$^+$ cells in suspensions of 7 day optic nerve are O-2A progenitor cells (Raff *et al.*, 1983b), some of them are GC$^+$ oligodendrocytes (Abney *et al.*, 1983) and it therefore seemed likely that many of the A2B5$^+$,vimentin-negative cells were oligodendrocytes, which, when mature, do not contain IFs (Peters *et al.*, 1976). In support of this hypothesis, we found that only 10% of the GC$^+$ oligodendrocytes in cell suspensions of 7 day optic nerve were vimentin positive and >95% of the A2B5$^+$ cells in 1 day optic nerve [which does not contain any GC$^+$ oligo-

Table II. Effect of RNA and protein synthesis inhibitors on O-2A progenitor cell differentiation

Time in culture (days)	Drug[a]	Proportion (%) of A2B5$^+$ cells expressing GFAP (in DMEM-FCS)	GC (in serum-free medium)
1	none	41 ± 3	14 ± 3
1	actinomycin D	<1	<1
1	cycloheximide	<1	<1
3	cycloheximide	62 ± 5	nd

[a]Seven day optic nerve cells growing on PLL-coated glass coverslips were treated with actinomycin D (0.5 μg/ml) for 1 h or cycloheximide (5 μg/ml) for 4 h.

Table III. Effects of cyclic nucleotides and NH_4Cl on O-2A progenitor cell differentiation in serum-free DMEM

Additive	Number of type 2 astrocytes[a] / Number of oligodendroctyes
None	0.05 ± 0.04
FCS (10%)	5.3 ± 2.4
dbc AMP (10^{-3} M)	0.03 ± 0.03
cholera toxin (200 ng/ml)	0.04 ± 0.03
dbc GMP (10^{-3} M)	0.04 ± 0.02
NH_4Cl (10 mM)	0.06 ± 0.04

[a]Seven day optic nerve cells were cultured on PLL-coated glass coverslips in serum-free DMEM for 3 days and then stained with anti-GC and anti-GFAP antibodies. The ratio of GC$^-$,GFAP$^+$ cells with a type 2 astrocyte morphology (putative type 2 astrocytes) to GC$^+$,GFAP$^-$ oligodendrocytes was determined by counting at least 200 labelled cells. Cells that were doubly labelled (GC$^+$,GFAP$^+$) made up <5% of the process-bearing cells and were not counted.

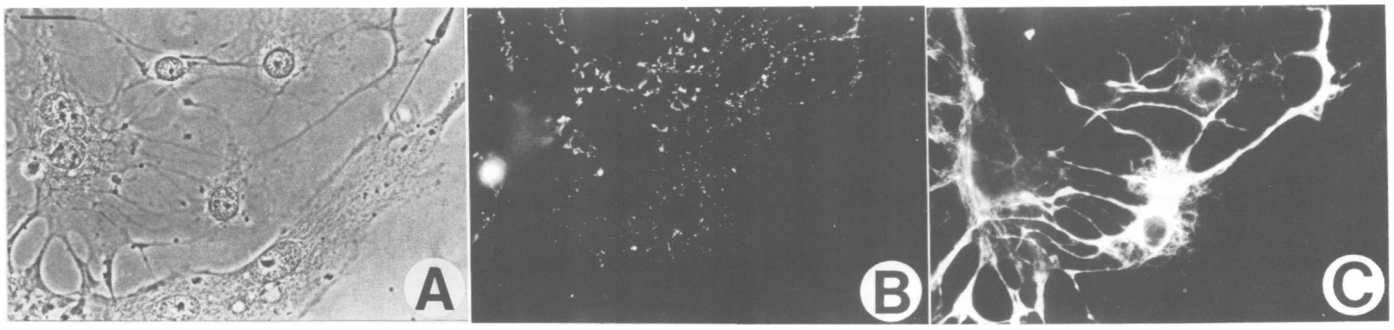

Fig. 3. Cytochalasin D does not block type 2 astrocyte development. Cultures of 7 day optic nerve cells were maintained for 3 days in DMEM-FCS in the presence of cytochalasin D (2 μg/ml) before they were labelled with A2B5 and anti-GFAP antibodies and viewed as described in Figure 1. Note that while the three type 2 astrocytes (A2B5$^+$,GFAP$^+$) are mononucleate, the other cells in the field (including a type 1 astrocyte) are multinucleate.

dendrocytes (R.H.Miller, S.David, E.R.Abney and M.C. Raff, in preparation)] were vimentin positive.

If O-2A progenitor cells contain vimentin filaments and mature oligodendrocytes do not, the former cells must lose their vimentin filaments during the course of differentiating into oligodendrocytes. We could demonstrate this loss in culture. When 7 day optic nerve cells were cultured in serum-free DMEM, >80% of the GC$^+$ cells were vimentin positive after 2 days, but <50% were vimentin positive after 4 days and these cells contained very little vimentin (Figure 4). On the other hand, when 7 day optic nerve cells were cultured in DMEM-FCS for 4 days, >95% of the A2B5$^+$ cells (almost all of which were type 2 astrocytes) remained strongly vimentin positive (not shown).

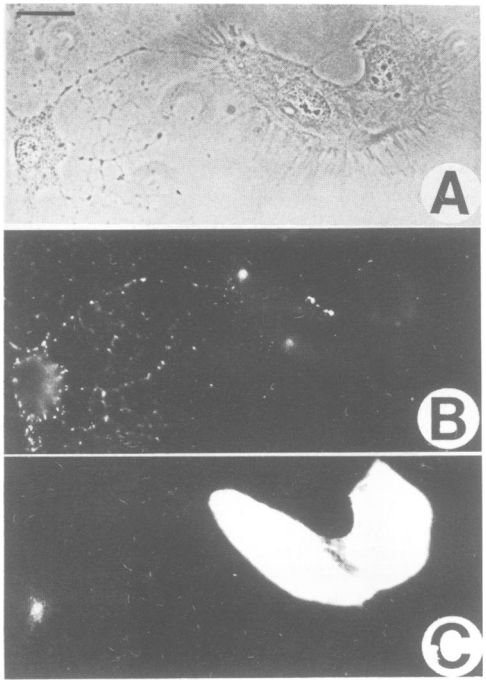

Fig. 4. A GC$^+$ oligodendrocyte with a small amount of residual vimentin. Optic nerve cells were treated with anti-GC antibody and complement and then cultured for 4 days in serum-free DMEM before they were labelled with anti-GC antibody and anti-vimentin antibodies and viewed as in Figure 1. The filamentous distribution of the vimentin staining in the two GC$^-$ flat cells is obscured by the photographic conditions required to visualize the residual vimentin in the oligodendrocyte.

The choice of differentiation pathway in culture is reversible for 1 or 2 days

To determine whether an O-2A progenitor cell that has begun to differentiate along one pathway can subsequently switch and differentiate along the other, 7 day optic nerve cells were first incubated in serum-free DMEM and then switched to DMEM-FCS, or *vice versa*. As can be seen in the upper half of Table IV, most O-2A progenitor cells that were grown in serum-free DMEM for 1 day before being switched to DMEM-FCS for 2 days, developed into GC$^-$,GFAP$^+$ type 2 astrocytes, and many others showed a mixed GC$^+$,GFAP$^+$ phenotype. On the other hand, most O-2A progenitor cells that were grown in serum-free DMEM for 2 days before being switched to DMEM-FCS for 2 days developed into GC$^+$,GFAP$^-$ oligodendrocytes. Thus, the majority of O-2A progenitor cells, initially cultured in serum-free DMEM, were able to become GFAP$^+$ if switched to DMEM-FCS after 24 h but not after 48 h.

As shown in the lower half of Table IV, when 7 day optic nerve cells were cultured in DMEM-FCS for 2 days and then switched to serum-free DMEM for 2 days, the great majority of O-2A progenitor cells became GC$^+$; while many developed into GC$^+$,GFAP$^-$ oligodendrocytes, most acquired a mixed GC$^+$,GFAP$^+$ phenotype. Similar results were obtained when cultures were treated with anti-GC antibody and complement just prior to switching to serum-free DMEM. Since <1 in 500 process-bearing cells was GC$^+$ immediately after such treatment, the great majority of the GC$^+$ cells that developed after the medium switch must have developed from GC$^-$ cells – presumably mainly from type 2 astrocytes, which constituted >85% of the process-bearing cells at the time of the switch. While most of the cells that acquired GC after the medium switch were weakly GFAP$^+$ (Figure 5), 10–20% did not contain detectable amounts of GFAP or vimentin. When 7 day optic nerve cells were cultured for 3 days in DMEM-FCS before being switched to serum-free DMEM for 2 or 3 days, >80% of the GFAP$^+$ astrocytes with a type 2 morphology were still GC$^-$, suggesting that, after 3 days in FCS, most type 2 astrocytes could no longer be induced to express GC.

The switch experiments described above examined the

Table IV. Reversibility of O-2A progenitor cell differentiation[a]

Medium	Proportion (%) of process-bearing cells that were		
	type 2 astrocytes (GC$^-$,GFAP$^+$)	oligodendrocytes (GC$^+$,GFAP$^-$)	mixed phenotype (GC$^+$,GFAP$^+$)
serum-free DMEM x 4 days	2 ± 2	95 ± 2	4 ± 1
serum-free DMEM x 1 day then DMEM-FCS x 2 days	53 ± 4	15 ± 3	33 ± 2
serum-free DMEM x 2 days then DMEM-FCS x 2 days	16 ± 2	64 ± 2	20 ± 3
DMEM-FCS x 4 days	92 ± 5	5 ± 3	3 ± 2
DMEM-FCS x 2 days then serum-free DMEM x 2 days	6 ± 1	40 ± 3	54 ± 2
DMEM-FCS x 3 days then serum-free DMEM x 3 days	82 ± 4	11 ± 2	7 ± 3

[a]Seven day optic nerve cells were cultured and subsequently labelled on PLL-coated glass coverslips. Only process-bearing cells were scored. The results for cells cultured for 3 days in serum-free DMEM or DMEM-FCS were indistinguishable from results for cells cultured for 4 days in the same medium.

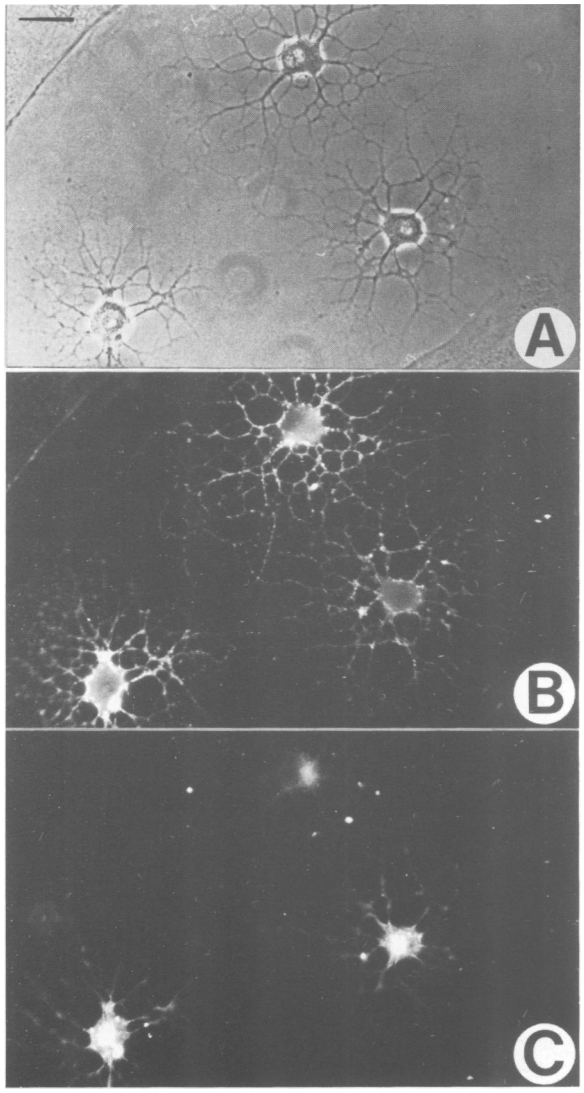

Fig. 5. GC^+ cells developing after a switch in culture medium. Optic nerve cells were cultured for 2 days in DMEM-FCS, then treated with anti-GC antibody and complement and then cultured in serum-free DMEM for 2 more days. The cells were labelled with anti-GC and anti-GFAP antibodies and viewed as in Figure 1. Note that the three GC^+ cells contain variable amounts of residual GFAP.

relative proportions of the different antigenic phenotypes of process-bearing cells grown on PLL-coated glass coverslips. Since the total numbers of each cell type were not assessed, the results could be misleading if selective cell death occurred in the cultures. To circumvent this problem, we repeated the switch experiments in a quantitative way, growing cells on culture dishes and removing them with trypsin and EDTA before counting and labelling them with antibodies. Such experiments confirmed the results described above (Table V).

To determine whether the switches in antigenic phenotype that occurred following the switch in culture medium were accompanied by appropriate morphological changes, we studied such 'switched' cells by immunogold electron microscopy. We have shown previously, using immunogold electron microscopy, that most O-2A progenitor cells acquire the ultrastructural morphology of fibrous astrocytes if cultured for 3 days in DMEM-FCS, and of oligodendrocytes if cultured for 3 days in serum-free DMEM (Raff *et al.*, 1983b). When 7 day optic nerve cells, which were grown in DMEM-FCS for 2 days followed by 2 days in serum-free DMEM, were labelled with anti-GC antibody followed by gold-conjugated anti-MIg and then examined by electron microscopy, the great majority of process-bearing cells were weakly GC^+ and had small numbers of IFs. These cells had a morphology that was intermediate between the GC^+,IF^- oligodendrocytes that developed in serum-free DMEM after 4 days and the GC^-,IF^+ type 2 astrocytes that developed in DMEM-FCS after 4 days (Figure 6). When cells were grown in DMEM-FCS for 2 days followed by 5 days in serum-free DMEM, most of the process-bearing cells were intensely GC^+ and were morphologically very similar to the oligodendrocytes that developed in 4 day cultures maintained in serum-free DMEM from the start (Figure 6). Similar results were obtained when the cultures were treated with anti-GC antibody and complement to kill the existing GC^+ oligodendrocytes prior to switching the medium.

Discussion

We showed previously that O-2A progenitor cells differentiate into type 2 astroctyes if cultured in DMEM-FCS and into oligodendrocytes if cultured in serum-free DMEM (Raff *et al.*, 1983b). Here we have shown that these bipotential glial progenitor cells differentiate rapidly in culture – some cells acquiring GFAP or GC within 1 day – and that the acqui-

Table V. Quantitating the reversibility of O-2A progenitor cell differentiation[a]

Culture medium	Numbers of cells per culture			
	type 1 astrocytes ($A2B5^-,GFAP^+$)	type 2 astrocytes ($A2B5^+,GFAP^+$)	oligodendrocytes (GC^+)	$GC^+,GFAP^+$ cells
serum-free DMEM x 4 days	4616 ± 2550	452 ± 172	8505 ± 652	419 ± 207
DMEM-FCS x 4 days	14 060 ± 4406	7280 ± 2816	1620 ± 535	264 ± 207
serum-free DMEM x 2 days then DMEM-FCS x 2 days	7315 ± 1916	1773 ± 408[b]	6258 ± 2332	1717 ± 521
DMEM-FCS x 2 days then serum-free DMEM x 2 days	8395 ± 2960	1858 ± 451[b]	8289 ± 2098	6684 ± 993

[a]Approximately 20 000 7 day optic nerve cells were plated in 60 μl of serum-free DMEM in PLL-coated Nunc culture dishes and after 15–20 min, 2 ml of the same medium was added and, where indicated, FCS was added to a final concentration of 10% (v/v). In some cases the medium was changed after 2 days. After 4 days, cells were removed from the dishes with trypsin-EDTA, counted in a haemocytometer and labelled in suspension as previously described (Raff *et al.*, 1983a, 1983b).
[b]Most of these $A2B5^+,GFAP^+$ cells were also GC^+ and therefore included among the $GC^+,GFAP^+$ cells.

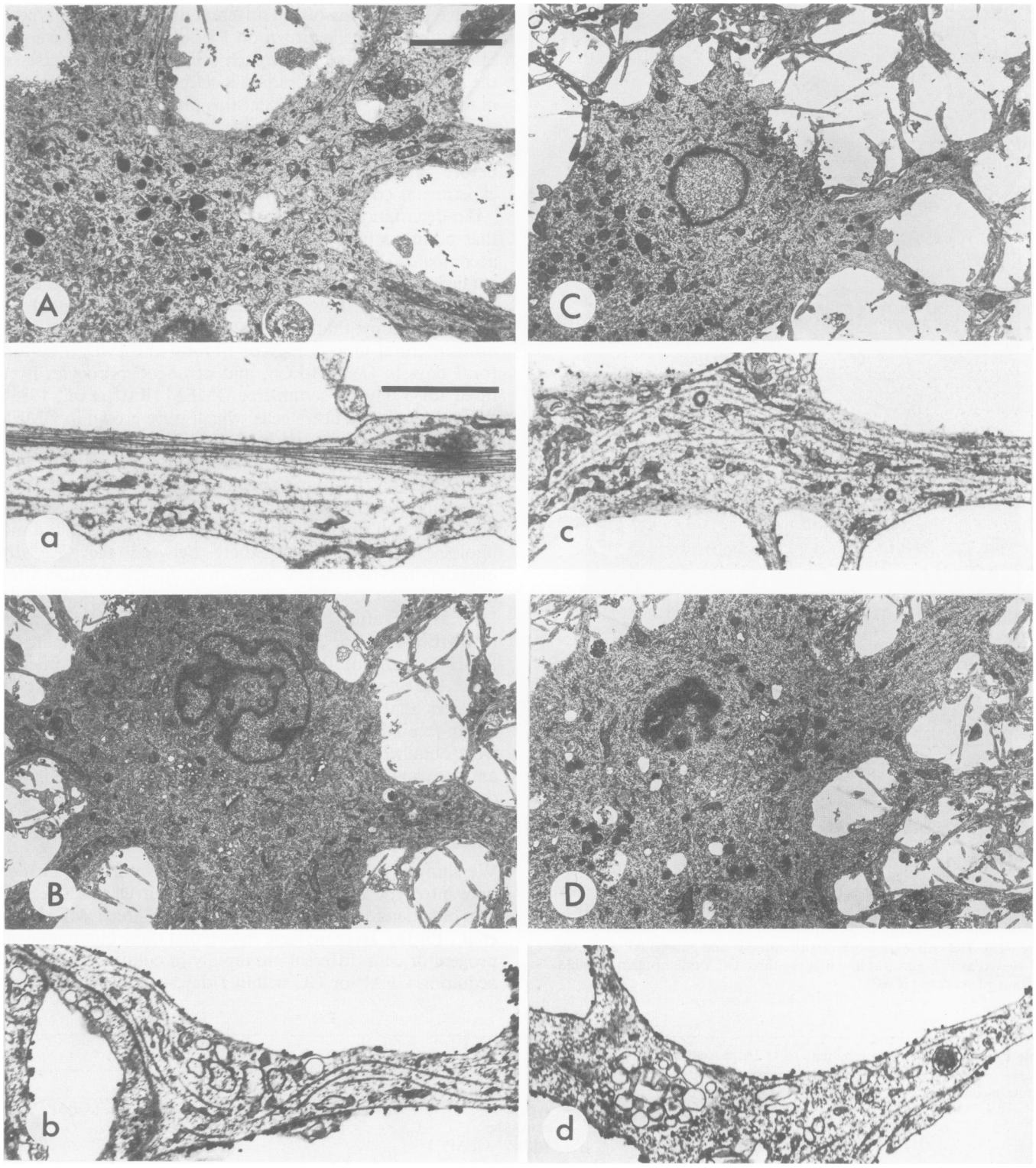

Fig. 6. Electron micrographs of cell bodies (capital letters) and processes (small letters) of cells in cultures of 7 day optic nerve maintained in DMEM-FCS for 4 days (**A** and **a**), serum-free DMEM for 4 days (**B** and **b**), DMEM-FCS for 2 days followed by serum-free DMEM for 2 days (**C** and **c**), and DMEM-FCS for 2 days followed by serum-free DMEM for 5 days (**D** and **d**). The cells were fixed in glutaraldehyde and then labelled with anti-GC antibody followed by G anti-MIg coupled to gold. In **C** and **D**, the cultures were treated with anti-GC antibody and complement prior to switching to serum-free DMEM. Note that the type 2 astrocyte in **A** (and **a**) has a relatively light cytoplasm, broad processes, dense bodies, bundles of glial filaments, few microtubules and is not labelled with anti-GC antibody, whereas the oligodendrocyte in **B** (and **b**) has a dense cytoplasm, finer processes, a large number of microtubules, no dense bodies or glial filaments and is heavily labelled by anti-GC antibody. The cell in **C** (and **c**) has an intermediate morphology with a few dense bodies, a mixture of glial filaments and microtubules and sparse anti-GC labelling, while the cell in **D** (and **d**) is very similar to the oligodendrocyte in **B**. More than 50 process-bearing cells were examined in each of the culture conditions and >80% had most of the characteristic features illustrated in the figure. Bar = 5 μm in **A**, **B**, **C** and **D** and 1 μm in **a**, **b**, **c** and **d**.

sition of either of these molecules requires both RNA and protein synthesis.

There are relatively few reported examples of normal progenitor cells that can be induced to differentiate in culture into either of two very different cell types. One is the avian limb bud mesenchymal cell that becomes (or remains) a fibroblast-like cell or a chondrocyte, depending on the culture conditions (Von der Mark, 1980; Solursh, 1983). In this case it has been shown that manipulating cell shape can greatly influence the choice of developmental pathway: manipulations that maintain the mesenchymal cells in a rounded configuration, such as treating the cells with the actin filament-disrupting drug cytochalasin D (Zanetti and Solursh, 1984) or culturing them on relatively non-adherent surfaces (Archer *et al.*, 1982), promote chondrocyte differentiation, while culture conditions that encourage the cells to adhere and flatten on the substrate inhibit this differentiation pathway and the cells instead adopt a fibroblast phenotype (Tsunematsu, 1979; Archer *et al.*, 1982; Swalla and Solursh, 1984). Similar observations have been reported on certain sublines of 3T3 cells that can be induced to form adipocytes in culture (Green, 1979). In this case, the induction of lipogenic enzymes seems to depend on the cells adopting a rounded configuration: the induction is inhibited by manoeuvres that promote cell adherence and spreading and this inhibition is reversed by cytochalasin D (Spiegelman and Ginty, 1983). Since O-2A progenitor cells tend to spread more and extend broader processes when cultured in DMEM-FCS than in serum-free DMEM (see Figures 1, 2 and 6), it seemed possible that FCS influences O-2A progenitor cell differentiation by increasing cell-substrate adherence and/or by affecting actin filaments. To assess this possibility we studied the effects of culturing the cells in DMEM-FCS on a relatively non-adherent substrate (bacteriological dishes) or in the continuous presence of cytochalasin D. Neither of these conditions encouraged oligodendrocyte development or prevented type 2 astrocyte development. Although many of the cells grown in cytochalasin D were multinucleate (resulting from the drug's inhibiting cytokinesis), including some type 2 astrocytes, other type 2 astrocytes were mononucleate, confirming our previous findings (Raff *et al.*, 1983b) that suggested that O-2A progenitor cells can differentiate in culture without a mandatory 'quantal' cell division (Holtzer, 1978).

If FCS does not induce the O-2A progenitor cell to develop into a type 2 astrocyte by influencing cell adherence or cell shape, how does it act? Since the relevant factor(s) in FCS seems to be macromolecular (Raff *et al.*, 1983b), it is likely that it acts by binding to cell-surface receptors (either on O-2A progenitor cells themselves or on some intermediary cells in the culture) that transduce the extracellular signal into an intracellular one. Our present results suggest that the transduction mechanism does not involve an increase in intracellular cyclic nucleotide levels, since dibutyryl cyclic AMP, cholera toxin [an irreversible activator of adenylate cyclase (Cassel and Pfeuffer, 1978)] and dibutyryl cyclic GMP, did not mimic the effect of FCS and had no appreciable influence on glial cell development in optic nerve cultures. Neither did NH_4Cl, which tends to increase intracellular pH (Hutchens *et al.*, 1939; Epel *et al.*, 1974), suggesting that the effects of FCS are not mediated in this way either.

It was of interest to study the expression of vimentin in the O-2A progenitor cell since one of the cells it gives rise to contains vimentin while the other does not; essentially all of the astrocytes in the optic nerve contain intermediate (glial) filaments composed of GFAP and vimentin (Shaw *et al.*, 1981), whereas oligodendrocytes are among the few vertebrate cell types that do not contain IFs (Peters *et al.*, 1976). It follows that either the O-2A progenitor cell contains IFs and loses them on developing into an oligodendrocyte, or the progenitor cell lacks IFs and acquires them on developing into a type 2 astrocyte. Our findings, and those of Schachner and her colleagues (Schachner *et al.*, 1984), indicate that the former sequence is the correct one: O-2A progenitor cells contained vimentin filaments and gradually lost them as they differentiated into oligodendrocytes in serum-free DMEM *in vitro*; in DMEM-FCS, the progenitor cells retained IFs on differentiating into type 2 astrocytes, but now the IFs were composed of GFAP as well as vimentin.

Perhaps the most interesting finding reported here is that the choice of differentiation pathway taken by an O-2A progenitor cell in culture is reversible, at least for a day or two. Many cells that had acquired GFAP and the morphological characteristics of type 2 astrocytes during 2 days in culture in DMEM-FCS, changed their developmental course when switched to serum-free DMEM and differentiated into GC^+ cells: many of these remained $GFAP^+$ for at least several days after the medium switch, while others became *bona fide* GC^+,$GFAP^-$,vimentin-negative oligodendrocytes. On the other hand, very few type 2 astrocytes acquired GC when the medium was switched from DMEM-FCS to serum-free DMEM after 3 rather than 2 days, suggesting that the commitment to astrocyte differentiation eventually becomes irreversible, at least under the conditions we studied. Differentiation along the oligodendrocyte pathway was also reversible but for a shorter period of time: whereas many of the O-2A progenitor cells developed into type 2 astrocytes when switched from serum-free DMEM to DMEM-FCS after 1 day, most remained GC^+,$GFAP^-$ oligodendrocytes when the medium was switched after 2 days. The molecular genetic mechanisms that underlie the initial binary choice of developmental pathway and its subsequent stabilization remain to be discovered.

Materials and methods

Cell suspensions and cultures

Cell suspensions were prepared form optic nerves of 7 day old Sprague-Dawley rats as previously described (Raff *et al.*, 1983a, 1983b) except that the nerves were cut into small pieces using iridectomy scissors, and the final incubation in trypsin and collagenase was carried out in the presence of 0.01% (w/v) EDTA, and the cells were washed in Minimal Eagle's medium containing 0.02 M Hepes buffer and 1% bovine serum albumin (Fraction V, Sigma) instead of 10% FCS. Cells were cultured either in Dulbecco's modified Eagle's medium containing 10% FCS (DMEM-FCS), or in DMEM supplemented with glucose, insulin, transferrin, albumin, progesterone, putrescine, selenium, thyroxine and tri-iodothyronine (serum-free DMEM) as previously described (Raff *et al.*, 1983b). Approximately 10 000 – 20 000 cells were plated in 20 μl of serum-free DMEM on either PLL-coated 13 mm glass coverslips or PLL-coated 35 mm Nunc tissue culture dishes, and after 15 – 30 min, 500 μl (for coverslips) or 2 ml (for dishes) of appropriate medium was added.

Drugs

Actinomycin D (Sigma) was dissolved in ethanol at a concentration of 1 mg/ml, further diluted to 100 μg/ml in water and used at a final concentration of 0.5 μg/ml. Cycloheximide (Sigma) was dissolved in water at a concentration of 1 mg/ml and used at a final concentration of 5 μg/ml. Cytochalasin D (Sigma) was dissolved in dimethylsulfoxide at a concentration of 1 mg/ml and used at a final concentration of 2 μg/ml. Dibutyryl cyclic GMP and dibutyryl cyclic AMP (Sigma) were dissolved in water to make 1 M solutions and were used at a final concentration of 10^{-3} M; they were added

every day to the cultures. Cholera toxin (Schwartz-Mann) was diluted in water and used at a final concentration of 200 ng/ml.

Killing cells with anti-GC antibody and complement

Seven day optic nerve cells in suspension or growing on PLL-coated glass coverslips were treated with 1 μl of monoclonal anti-GC antibody [ascites fluid (Ranscht *et al.*, 1982)] diluted in 30 μl of agarose-absorbed rabbit complement (Cohen and Schlesinger, 1970) and 270 μl of either Eagle's Minimal Essential Medium with 0.02 M Hepes buffer (for cells in suspension) or serum-free DMEM (for cells on coverslips) for 45 min at 37°C. Cells in suspension were incubated in a waterbath while cells on coverslips were incubated in a CO_2 incubator. After washing, the cells were cultured on PLL-coated coverslips in serum-free DMEM.

Immunofluorescence assays

All the antibodies used in this study have been described previously: monoclonal anti-GC antibody (Ranscht *et al.*, 1982) and the A2B5 monoclonal antibody (Eisenbarth *et al.*, 1979) were used as ascites fluid diluted 1:500; rabbit anti-GFAP antiserum (Pruss, 1979) was diluted 1:1000 and rabbit anti-vimentin serum (Hynes and Destree, 1978) was diluted 1:250. The monoclonal antibodies were visualized with goat anti-mouse Ig antibodies conjugated to rhodamine (G anti-MIg-Rd, Cappel) or with class-specific anti-IgM-Rd for A2B5 (Nordic, 1:80) or anti-IgG3 antibodies conjugated to fluorescein for anti-GC antibody (anti-IgG3-Fl, Nordic, 1:100). The rabbit antisera were visualized with sheep anti-RIg-Fl (Wellcome, 1:100) absorbed with mouse IgG coupled to Sepharose 4B.

Cells in suspension, on glass coverslips or on culture dishes, were double labelled with A2B5 or anti-GC and then, after acid-alcohol fixation, with anti-GFAP or anti-vimentin antibodies as previously described (Raff *et al.*, 1983a). Cells on coverslips were double labelled with anti-GC and A2B5 antibodies by incubating the cells in anti-GC antibody followed by anti-IgG3-Fl and then in A2B5 antibody followed by anti-IgM-Rd. All cells were eventually fixed in acid-alcohol, mounted in glycerol containing 0.1% (w/v) *p*-phenylenediamine to prevent fading (Johnson *et al.*, 1982), examined in a Zeiss Universal fluorescence microscope and photographed as previously described (Raff *et al.*, 1979).

Immunogold electron microscopy

Cells on glass coverslips or culture dishes were pre-fixed in 1% glutaraldehyde for 10 min, incubated in anti-GC antibody followed by G anti-MIg coupled to gold (Janssen, 1:5), post-fixed in 2% glutaraldehyde, osmicated and stained with 0.5% aqueous uranyl acetate, dehydrated through graded alcohols and embedded in Epon 812 as previously described (Raff *et al.*, 1983b). Thin sections were cut parallel to the substrate and stained with uranyl acetate and lead citrate before examination on a Jeol 100 CXII electron microscope.

References

Abney,E.R., Williams,B. and Raff,M.C. (1983) *Dev. Biol.*, **100**, 166-171.

Archer,C.W., Rooney,P. and Wolpert,L. (1982) *Cell Differ.*,, **11**, 245-251.

Bignami,A., Eng,L.F., Dahl,D. and Uyeda,C.T. (1972) *Brain Res.*, **43**, 429-435.

Cassel,D. and Pfeuffer,T. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2669-2673.

Cohen,A. and Schlesinger,M. (1970) *Transplantation*, **10**, 130-134.

Eisenbarth,G.S., Walsh,F.S. and Nirenberg,M. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4913-4917.

Epel,D., Steinhardt,R., Humphreys,T. and Masia,D. (1974) *Dev. Biol.*, **40**, 245-255.

Green,H. (1979) *INSERM*, **87**, 15-24.

Holtzer,H. (1978) in Lord,B., Potten,C. and Cole,P. (eds.), *Stem Cells and Tissue Homeostasis*, Cambridge University Press, Cambridge, pp. 1-28.

Hutchens,J.O., Krahl,M.E. and Clowes,G.H.A. (1939) *J. Cell. Comp. Physiol.*, **14**, 313-325.

Hynes,R.O. and Destree,A.T. (1978) *Cell*, **13**, 151-163.

Johnson,G.D., Davidson,R.S., McNamee,K.C., Russell,G., Goodwin,D. and Holborow,E.J. (1982) *J. Immunol. Methods*, **55**, 231-242.

Miller,R.H. and Raff,M.C. (1984) *J. Neurosci.*, **4**, 585-592.

Peters,A., Palay,S.L. and Webster,H.de F. (eds.), (1976) in *The Fine Structure of the Nervous System: The Neurons and Supporting Cells*, W.B. Saunders Co., Philadelphia, pp. 248-250.

Pruss,R. (1979) *Nature*, **280**, 688-690.

Raff,M.C., Mirsky,R., Fields,K.L., Lisak,R.P., Dorfman,S.H., Silberberg, D.H., Gregson,N.A., Liebowitz,S. and Kennedy,M. (1978) *Nature*, **274**, 813-816.

Raff,M.C., Fields,K.L., Hakomori,S., Mirsky,R., Pruss,R.M. and Winter,J. (1979) *Brain Res.*, **174**, 283-308.

Raff,M.C., Abney,E.A., Cohen,J., Lindsay,R. and Noble,M. (1983a) *J. Neurosci.*, **3**, 1289-1300.

Raff,M.C., Miller,R.H. and Noble,M. (1983b) *Nature*, **303**, 390-396.

Ranscht,B., Clapshaw,P.A., Price,J., Noble,M. and Seifert,W. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2709-2713.

Schachner,M., Faissner,A., Kruse,J., Lindner,J., Meier,D.H., Rathjen,F.G. and Wernecke,H. (1984) *Cold Spring Harbor Symp. Quant. Biol.*, **48**, 557-568.

Shaw,G., Osborn,M. and Weber,K. (1981) *Eur. J. Cell Biol.*, **26**, 68-82.

Solursh,M. (1983) in Kall,B. (ed.), *Cartilage*, vol. **2**, Academic Press, NY, pp. 121-141.

Spiegelman,B.M. and Ginty,C.A. (1983) *Cell*, **43**, 657-666.

Swalla,B.J. and Solursh,M. (1984) *Differentiation*, **26**, 42-48.

Tsunematsu,Y. (1979) *Dev. Growth Differ.*, **21**, 437-444.

Von der Mark,K. (1980) *Curr. Top. Dev. Biol.*, **14**, 199-223.

Zanetti,N.C. and Solursh,M. (1984) *J. Cell Biol.*, in press.

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