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Purified astrocytes promote the *in vitro* division of a bipotential glial progenitor cell

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Optic nerves of neonatal rats contain a bipotential glial progenitor cell which can be induced by tissue culture conditions to differentiate into either an oligodendrocyte (the myelin-forming cell of the CNS) or a type 2 astrocyte (an astrocyte population found only in the myelinated tracts of the CNS). In our previous studies most oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells differentiated within 3 days *in vitro* with relatively little division of the progenitors or their differentiated progeny. We have now found that the O-2A progenitors are stimulated to divide in culture by purified populations of type 1 astrocytes, another glial cell-type found in the rat optic nerve. This cell-cell interaction appears to be mediated by a soluble factor(s) and results in the production of large numbers of both progenitor cells and oligodendrocytes. As type 1 astrocytes are the major glial cell-type in the optic nerve when oligodendrocytes first begin to be produced in large numbers *in vivo*, our results suggest that this astrocyte subpopulation may play an important role in expanding the oligodendrocyte population during normal development.

Key words: glial progenitors/mitogens/oligodendrocytes/astrocytes/CNS development

Introduction

The development of the central nervous system (CNS) from the neural tube is characterized by, among other events, massive increases in cell numbers. Attempts to understand the regulation of cellular division in the CNS *in vivo* are faced with the immense complexity of this tissue, which contains a variety of cell-types which divide and differentiate according to separate schedules under the regulation of unknown cell-cell interactions. To simplify the study of this complex process, we have been using *in vitro* preparations to examine the regulation of division of a glial progenitor cell present in the rat optic nerve, the simplest part of the central nervous system (Raff *et al.*, 1983a, 1983b). As the optic nerve contains no neuronal cell bodies, this preparation allows us to examine glial development in the absence of neurons. The simplicity of this tissue offers us the hope of understanding the factors controlling development in at least one portion of the CNS.

In previous studies, we have found that the rat optic nerve contains three populations of macroglial cells: oligodendrocytes and two types of astrocytes (see Table I). Type 1 astrocytes are found throughout the CNS; these cells are labelled by antisera against glial fibrillary acidic protein (GFAP), an astrocyte-specific marker (Raff *et al.*, 1979), but do not bind either tetanus toxin or the monoclonal antibody A2B5 [which bind to specific gangliosides (Van Heyningen, 1963; Eisenbarth *et al.*, 1979)]. Type 1 astrocytes are the first differentiated glial cell found in the optic nerve (Miller and Raff, 1984; Raff *et al.*, 1984). In contrast, type 2 astrocytes appear to be largely restricted to the white matter tracts of the CNS. They appear later in development than type 1 astrocytes and become a significant population in the optic nerve ~3 weeks after birth; type 2 astrocytes are the major astrocyte-type in the adult optic nerve (Miller and Raff, 1984). Type 2 astrocytes bind both A2B5 and tetanus toxin, and are also GFAP$^+$. These two types of astrocytes appear to represent two distinct lines of glial development, which may correspond to protoplasmic (= type 1) and fibrous (= type 2) astrocytes (Raff *et al.*, 1983a; Miller and Raff, 1984).

In vitro studies have indicated that oligodendrocytes, the myelin forming cells of the CNS, and type 2 astrocytes develop from a common bipotential progenitor cell, which has been identified in cultures of neonatal rat optic nerve (Raff *et al.*, 1983b). When grown *in vitro*, this bipotential progenitor rapidly differentiates into either an oligodendrocyte or a type 2 astrocyte, depending upon the tissue culture medium. In cultures of optic nerve, the oligodendrocyte-type 2 astrocyte progenitors (O-2A progenitors) and their differentiated progeny undergo relatively little cell division; thus the optic nerve cultures are depleted of progenitors as differentiation proceeds (Raff *et al.*, 1983b). This pattern of *in vitro* development differs markedly from events *in vivo*, where generation of a complete complement of oligodendrocytes and type 2 astrocytes takes several weeks, is associated with cell division, and progenitors are still found at least 2 weeks after the first appearance of oligodendrocytes (Skoff *et al.*, 1976a, 1976b; Raff *et al.*, 1983b). Thus, some signal missing from our previous optic nerve cultures, but present in the optic nerve *in vivo*, is necessary to promote maintenance and expansion of the O-2A progenitor population. The major cell-types present in the optic nerve *in vivo* during early expansion of the O-2A progenitor population are neurons (or, more accurately, axons) and type 1 astrocytes. *In vivo* studies have indicated that O-2A progenitors will synthesize DNA (and presumably divide) in the absence of axons (David *et al.*, 1984), thus raising the possibility that type 1 astrocytes may stimulate division of O-2A progenitors *in vivo*.

We have now discovered that purified type 1 astrocytes promote the *in vitro* division of the O-2A progenitor, leading to increasing numbers of both O-2A progenitors and oligodendrocytes. The effects of type 1 astrocytes on O-2A progenitors appear to be mediated by a soluble factor, or factors, secreted into chemically-defined medium. Thus, a defined glial cell-type from the central nervous system stimulates the division of a progenitor cell from a separate glial lineage. These results suggest that type 1 astrocytes may play a role in expanding the O-2A lineage during development and offer an explanation for some of the markedly different patterns of division and differentiation seen in the optic nerve O-2A lineage *in vivo* and *in vitro*.

Table I. Antigenic phenotypes of optic nerve cells in the 7-day-old rat

Cell type	Antigenic phenotype	% of total optic nerve cells in dissociated cell suspensions*
Type 1 astrocyte	GFAP$^+$, RAN-2$^+$ A2B5$^-$, tetanus toxin$^-$ (Raff *et al.*, 1983a, 1984)	8
Type 2 astrocyte	GFAP$^+$, A2B5$^+$, tetanus toxin$^+$ RAN-2$^-$ (Raff *et al.*, 1983a, 1984)	<0.1
Oligodendrocyte	GalC$^+$, GFAP$^-$ (Raff *et al.*, 1978, 1979)	7
Oligodendrocyte-type 2 astrocyte (O-2A) progenitors	A2B5$^+$, tetanus toxin$^+$ GalC$^-$, GFAP$^-$ (Raff *et al.*, 1983a, 1983b, 1984)	28

*These percentages are derived from Raff *et al.* (1983a, 1983b), and from recent studies by R.Miller, S.David, R.Patel, E.Abney and M.Raff (in preparation). 57% of the cells in the suspensions of optic nerve from 7-day-old rats are not presently identifiable.
GalC, galactocerobroside; GFAP, glial fibrillary acidic protein; RAN-2, rat neural antigen-2 (Bartlett *et al.*, 1981); A2B5, monoclonal antibody A2B5.

Results

Cell-type identification

The cell populations of interest were identified using previously described antibodies (see Table I). The antigenic phenotypes of type 1 and type 2 astrocytes have been described in the Introduction. Oligodendrocytes were labelled by monoclonal anti-galactocerebroside antibodies (Raff *et al.*, 1978; Ranscht *et al.*, 1982). The bipotential O-2A progenitor is A2B5$^+$, but GFAP$^-$ and GalC$^-$ (Raff *et al.*, 1983b). We have previously shown (Raff *et al.*, 1983b) that this cell-type can be induced to develop *in vitro* into an oligodendrocyte if grown in chemically-defined medium and into a type 2 astrocyte if grown in the presence of fetal sera. In cultures prepared from the optic nerves of 7-day-old rats, the great majority of the A2B5$^+$ cells appear to be O-2A progenitors (Raff *et al.*, 1983b).

Type 1 astrocytes stimulate DNA synthesis in O-2A progenitors

When optic nerve cultures, prepared as described previously (Raff *et al.*, 1983b), were plated onto poly-L-lysine (PLL) coated glass coverslips and grown in serum-free defined medium plus hormone supplements (Raff *et al.*, 1983b; Bottenstein and Sato, 1979), only 4–8% of O-2A progenitors incorporated [^{3}H]thymidine when pulsed for 20 h during the first 24 or 48 h in culture (Table II, and Raff *et al.*, 1983b). The great majority of O-2A progenitors disappeared within 72 h as they differentiated into oligodendrocytes (Raff *et al.*, 1983b). In contrast, when optic nerve cells were plated onto monolayers of purified type 1 astrocytes, or plated onto PLL-coated coverslips and grown in defined medium which had been conditioned for 24 h by type 1 astrocytes (Astro-CM), 60–70% of the O-2A progenitors incorporated [^{3}H]thymidine during a 20-h pulse (Table II). Up to 5% of the GalC$^+$ oligodendrocytes were also labelled with [^{3}H]thymidine in these cultures (data not shown). To determine whether this incorporation of label by oligodendrocytes represented DNA synthesis in oligodendrocytes or differentiation of oligodendrocytes from radio-labelled O-2A progenitors, we pulsed separate cultures with [^{3}H]thymidine for 6 h, so that it was unlikely that O-2A progenitors would differentiate into oligodendrocytes and express GalC during the time of the pulse. Virtually no label was incorporated into oligodendrocytes during the 6-h pulse period, although labelling of O-2A progenitors was proportionately comparable with that seen with 20-h pulses (Table II). Therefore, the great majority of any dividing cells in this lineage were likely to be progenitors and not oligodendrocytes. In addition, cells with the antigenic phenotype of O-2A progenitors (i.e., A2B5$^+$GalC$^-$GFAP$^-$) were detectable at both 3 days and 10 days after plating when optic nerve cells were grown on purified type 1 astrocytes or in Astro-CM; no such progenitor-like cells were found in control cultures. Thus, in contrast to development in control cultures, growth of optic nerve cells on type 1 astrocytes, or in Astro-CM, promoted DNA synthesis in O-2A progenitors and promoted the maintenance of cells with the antigenic phenotype of O-2A progenitors for extended periods in culture.

Table II. Stimulation of DNA synthesis in oligodendrocyte-type 2 astrocyte progenitors by type 1 astrocytes

Length of [^{3}H]thymidine pulse	Cell type (antigenic phenotype)	% of O-2A progenitors or oligodendrocytes with radiolabelled nuclei		
		Defined medium	Astrocyte-conditioned medium	On astrocyte monolayers
20 h	O-2A progenitors (A2B5$^+$ GFAP$^-$ GalC$^-$)	8.7 ± 1.4	63.3 ± 4.7	67.9 ± 4.4
6 h	O-2A progenitors	3.7 ± 0.5	37.7 ± 1.6	23.4 ± 1.7
6 h	oligodendrocytes (GalC$^+$ GFAP$^-$)	<1	<1	<1

Optic nerve cells were plated at 10 000 cells/coverslip and grown in defined medium, astrocyte-conditioned medium, or on monolayers of purified type 1 astrocytes. Cultures were grown for 18 h, after which 1 μCi of [^{3}H]thymidine was added to each culture for either 20 h or 6 h. Comparable results were found when [^{3}H]thymidine was added for a 20-h pulse immediately after the optic nerve cells were plated. When the radiolabelling period was over, cultures were labeled with A2B5 and anti-GalC antibodies, followed by rhodamine-conjugated anti-MIgM and fluorescein-conjugated anti-MIgG_3. Alternatively, cultures were labeled sequentially with either A2B5 or anti-GalC and rhodamine-conjugated anti-MIg, followed by anti-GFAP and fluorescein-conjugated anti-RIg (see Materials and methods) to confirm that the A2B5$^+$ GalC$^-$ cells were also GFAP$^-$. The percentage of cells with radio-labelled nuclei was then determined for O-2A progenitors and for oligodendrocytes. At least 200 cells/cover-slip on four or more cover-slips were counted. Mean ± SEM.

Oligodendrocyte and O-2A progenitor numbers increase in vitro

Stimulation of DNA synthesis in cultures of rat optic nerve cells grown on type 1 astrocytes was associated with marked increases in the number of both O-2A progenitor-like cells and oligodendrocytes. These increases were most easily quantitated when small numbers of optic nerve cells (1000 cells/cover slip) were plated onto monolayers of type 1 astrocytes. Increases were calculated by comparing the total number of cells in the O-2A lineage on days 3 and 10 with the number of O-2A progenitors present on day 1 (Table III). This particular comparison was made because (i) experiments thus far indicate that all oligodendrocytes differentiate from A2B5$^+$ precursors (Abney *et al.*, 1983; Raff *et al.*, 1983b) and (ii) few, if any, oligodendrocytes were synthesizing DNA

Table III. Growth of optic nerve cells on monolayers of type 1 astrocytes causes increases in the numbers of O-2A progenitor-like cells and oligodendrocytes

Cell type	Days in culture		
	1	3	10
O-2A progenitors	64 ± 2	125 ± 31	778 ± 41
Oligodendrocytes	34 ± 5	198 ± 17	754 ± 87
Percentage increase of O-2A progenitors and oligodendrocytes from day 1		451%	2300%

Optic nerve cells were plated onto monolayers of Type 1 astrocytes as for the experiments in Table II, except that only 1000 cells were plated per cover-slip. Cultures were labelled with A2B5 and anti-GalC antibodies, and all antibody-labelled cells were counted on each cover-slip. The percentage increase from day 1 was calculated by taking the total day 3 or day 10 values for [progenitors + oligodendrocytes], subtracting the number of oligodendrocytes present on day 1 (i.e., 34) and dividing this total by the number of progenitors found on day 1 (i.e., 64). Values are mean ± SEM for four cover-slips.

(and were therefore not dividing) in the present experiments and thus could not have contributed in a major way to the *in vitro* expansion of this lineage. We found 4- to 5-fold increases in numbers of cells in the O-2A lineage over the 48-h period of day 1 to day 3, suggesting a generation time of <24 h. Numbers of oligodendrocytes and O-2A progenitor-like cells continued to increase for at least another week, increasing a further 10-fold over the numbers of progenitor-like cells present on day 3. Thus, there was a 23-fold expansion over 10 days from initial populations of O-2A progenitors to day 10 levels of (O-2A progenitor-like cells + oligodendrocytes).

O-2A progenitors give rise to O-2A progenitor-like cells

Having found that type 1 astrocytes promoted DNA synthesis in O-2A progenitors and expansion of the O-2A lineage *in vitro*, we sought to determine whether the increasing numbers of O-2A progenitor-like cells were themselves the progeny of the O-2A progenitors in the original optic nerve suspension or instead arose from the large proportion of A2B5$^-$ cells present in the optic nerve at this stage. Two types of experiments were carried out. Firstly, freshly prepared suspensions of optic nerve cells were treated with A2B5 antibody plus complement to kill all the O-2A progenitors in the original suspension; the remaining cells were then grown on monolayers of type 1 astrocytes or in Astro-CM. No O-2A progenitor-like cells arose in these cultures when studied after 1 or 2 days *in vitro*; these results indicate that the original O-2A progenitors were necessary for the generation of more cells with the same antigenic phenotype for at least the same time period as our DNA synthesis experiments. Results indicating that O-2A progenitors were directly producing cells of the same antigenic phenotype were obtained by pulsing fresh suspensions of optic nerve cells with A2B5 antibody for 30 min, washing out the unbound antibody, and growing the A2B5-labelled cells in Astro-CM for 24 or 48 h while pulsing cultures for 20 h with [^{3}H]thymidine (Figure 1, Table IV). At the end of the [^{3}H]thymidine pulse, residual antibody A2B5 was visualised with a layer of rhodamine-conjugated anti-mouse immunoglobulin (Ig); cultures were then labelled with a further layer of A2B5, followed by fluorescein-conjugated anti-mouse Ig. Thus any cell which was A2B5$^+$ after the period of culture, which had also been A2B5$^+$ in the original prepara-

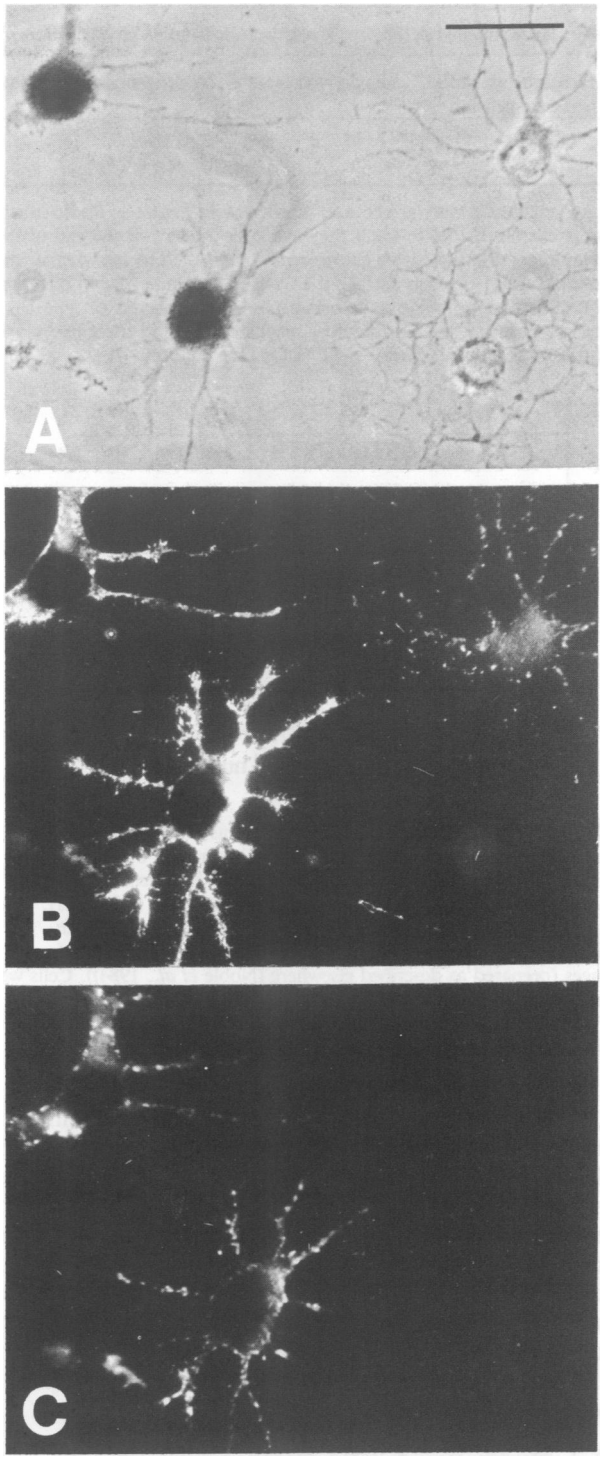

Fig. 1. Freshly dissociated optic nerve cells were labeled with A2B5 antibody (ascites, 1:200) for 30 min at 37°C. Cells were washed and plated onto PLL-coated cover-slips or astrocyte monolayers as in Table II. Cells on cover-slips were grown in Astro-CM, which was added fresh again after 24 h. Some cultures were pulsed with [^{3}H]thymidine for the first 20 h after plating, and then labeled sequentially with anti-MIg-Rd, A2B5 and anti-MIg-F1. Other cultures were allowed to first grow for 24 h before [^{3}H]-thymidine was added for 20 h, after which cultures were labeled with antibodies. The photo-micrographs are of cultures grown in Astro-CM and labeled with [^{3}H]thymidine during the last 20 h of a 44-h growth period. **(A)** Phase. Two cells have labelled nuclei. **(B)** Fluorescein. Three of the cells are A2B5$^+$, one of them only weakly. **(C)** Rhodamine. Both [^{3}H]thymidine$^+$, A2B5$^+$ cells have residual A2B5 on their surface, showing that they are derived from O-2A progenitors in the original optic nerve suspension. Bar = 20 μm.

Table IV. Radiolabelled A2B5$^+$ cells are derived from O-2A progenitors

% of radiolabelled A2B5$^+$ cells derived from O-2A progenitors in the initial optic nerve suspension	
Day 1	Day 2
77 ± 3%	66 ± 2%

Optic nerve cultures were grown and labelled as in Figure 1. Radio-labelled cells were examined first to see if they had been A2B5$^+$ at the end of the [^{3}H]thymidine pulse (i.e., were fluorescein$^+$), and then to see whether they had residual A2B5 on their surface (i.e., were rhodamine$^+$). No difference was found between growing optic nerve cells on monolayers of type 1 astrocytes and in Astro-CM; therefore results obtained in these two types of cultures were pooled. Mean ± SEM for nine cultures.

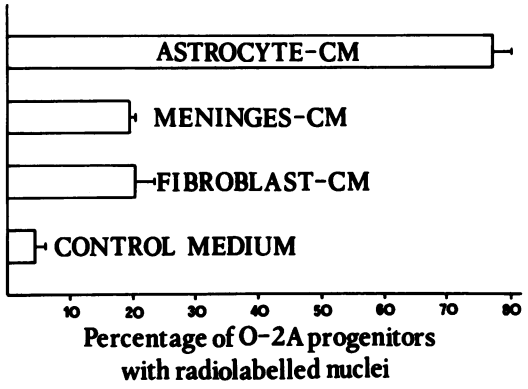

Fig. 2. Fibroblasts and meningeal cells are not as effective as type 1 astrocytes at promoting incorporation of [^{3}H]thymidine by O-2A progenitors *in vitro*. Optic nerve cells were plated at a density of 10 000 cells/cover-slip on PLL-coated cover-slips and grown as in Table II, except that some cultures were grown in defined medium which had been conditioned by confluent cultures of skin fibroblasts or astrocyte-free meninges (prepared as described elsewhere (Noble *et al.*, 1984)). Cells were grown for 20 h, at which time 1 μCi [^{3}H]thymidine was added to each well. After a further 24 h cultures were stained with antibodies, processed for autoradiography and scored as in Table II. The values are means ± SEM for triplicate cover-slips. CM = conditioned medium.

tion, would have been labelled with both rhodamine and fluorescein. Of those radio-labelled cells which were A2B5$^+$ on days 1 or 2, 77% and 66%, respectively, were found to express residual A2B5 antibody on their surfaces and therefore were derived from cells which had been A2B5$^+$ in the original optic nerve suspension. These experiments indicate that the majority of progenitor-like cells developing in culture, at least during the first 48 h, were derived from O-2A progenitors in the original optic nerve suspension and suggest that promotion of O-2A progenitor division leads to the generation of more O-2A progenitors.

Type 1 astrocytes are more effective than fibroblasts and meningeal cells at promoting DNA synthesis in O-2A progenitors

Fibroblasts and astrocyte-free meningeal cells, the most likely contaminants of our type 1 astrocyte cultures, were significantly less effective than type 1 astrocytes at promoting DNA synthesis in cells with the O-2A progenitor antigenic phenotype (Figure 2). This indicates that the growth-promoting activity we have ascribed to type 1 astrocytes was unlikely to be the property of the small percentage of non-astrocytes in the cultures and suggests that astrocytes may have a special ability to promote progenitor cell division. Even in the presence of fetal calf serum, which induces the O-2A progenitor to become a type 2 astrocyte (Raff *et al.*, 1983b), type 1 astrocytes still promoted division of O-2A progenitors and maintenance of cells with the O-2A progenitor phenotype for extended periods in culture (M.Noble, K.Murray and G.McKhann, in preparation).

Discussion

By studying the simplest part of the central nervous system, the optic nerve, and utilising *in vitro* and *in vivo* studies, cell-type specific markers and manipulation of cellular populations in culture, it has been possible to obtain two new insights into development of the CNS glia. Firstly, astrocytes develop from at least two distinct glial lineages which give rise separately to type 1 and type 2 astrocytes. Secondly, type 2 astrocytes and oligodendrocytes develop, in optic nerve cultures, from a common bipotential O-2A progenitor. One of the important next questions to be answered in our attempts to understand how the optic nerve develops, is to identify the means by which the O-2A glial lineage is expanded during development.

From the results presented here we conclude that type 1 astrocytes stimulate the multiplication of O-2A progenitors, the progenitor cells which give rise to oligodendrocytes and type 2 astrocytes. Promotion of division leads to the generation of increasing numbers of both O-2A progenitors and oligodendrocytes. It has been found previously that oligodendrocytes can develop in optic nerve cultures in the absence of neurons (Abney *et al.*, 1983; Raff *et al.*, 1983b). The continually increasing numbers of oligodendrocytes seen in our cultures indicates that neurons are also not required for stimulating the division of oligodendrocyte progenitors or for the generation of oligodendrocytes from a population of dividing progenitors *in vitro*. These results agree with *in vivo* observations (Privat *et al.*, 1981; David *et al.*, 1984), although there may also be *in vitro* conditions where GalC$^-$ oligodendrocyte precursors do not fully differentiate into GalC$^+$ oligodendrocytes (D.Meier *et al.*, in preparation). We do not yet know what factors play a role in triggering the differentiation of progenitors into oligodendrocytes.

Medium conditioned by type 1 astrocytes was as effective at promoting DNA synthesis in O-2A progenitors as growth on monolayers of purified type 1 astrocytes; thus, this effect appears to be mediated by a soluble factor or factors. Our initial experiments have indicated that the active factor(s) in the astrocyte-conditioned medium can be concentrated 1000-fold and stored at −20°C without losing activity (M.Noble, K.Murray, P.Stroobant and M.Waterfield, in preparation). This indicates that astrocytes are secreting the active agent(s), as opposed to inactivating a toxic component of the medium. As the O-2A progenitor mitogen(s) secreted by type 1 astrocytes is effective in serum-free medium, resists freezing and thawing and can be concentrated, we are presently attempting to identify the active molecules(s).

The results presented here offer a possible explanation for the contrasting patterns previously found for division and development of the O-2A lineage *in vivo* and *in vitro* (see Introduction). The potential relevance of these observations for *in vivo* development is particularly indicated by two recent findings: firstly, elimination of optic nerve axons, by neonatal optic nerve transection, does not alter [^{3}H]thymidine incorporation by O-2A progenitors *in vivo* (David *et al.*, 1984). Thus, axons are not necessary to maintain normal levels of DNA synthesis (and presumably division) in O-2A progenitors *in situ*. Secondly, although it has long been known that

astrocyte differentiation precedes oligodendrocyte differentiation in the rat optic nerve (Skoff *et al.*, 1976a, 1976b), it has only recently been found that the first astrocyte population to appear in the optic nerve is composed of cells with the antigenic phenotype of type 1 astrocytes (Miller and Raff, 1984). The type 1 astrocytes remain as the major astrocyte population in the rat optic nerve for at least the first 3 weeks after birth (Miller and Raff, 1984), that time period which is the peak period for genesis of oligodendrocytes *in vivo* (Skoff *et al.*, 1976a, 1976b). In fact, in the intact optic nerve, type 1 astrocytes may account for up to one third of the total cells; the type 1 astrocytes are disproportionately lost from optic nerve preparations during dissociation and thus form a lower percentage of the optic nerve cultures (R.Miller, S.David, R. Patel, E.Abney and M.Raff, in preparation). The lack of effect of enucleation on O-2A progenitor DNA synthesis *in situ*, and the early appearance of type 1 astrocytes in the optic nerve, together raise the possibility that stimulation of O-2A progenitor division *in situ* may be a function of type 1 astrocytes. As type 1 astrocytes are one of the major cell types in the CNS, it seems likely that interactions similar to those we have described here might play a role in a normal CNS development.

Materials and methods

Preparation of purified astrocytes

Purified type 1 astrocytes were prepared by a modification of previous procedures (Noble *et al.*, 1984). Cerebral cortex cultures from 1–2 day old Sprague Dawley rats, plated at a density of 2 x 10^7 cells per Falcon Flask No. 3024 (75 cm^2 surface area), were grown *in vitro* for 7–9 days. Flasks were then shaken overnight on a rotary platform at 37°C. The cells not removed by this procedure were 85–95% astrocytes, as judged by labelling with antiserum to glial fibrillary acidic protein (GFAP), a cell-type specific marker for astrocytes. Cultures were then pulsed twice for 2 days/pulse with fresh medium containing cytosine arabinoside (AraC, 2 x 10^{-5} M) to kill dividing cells. After AraC treatment, cells were removed from flasks with 0.25% trypsin and 0.02% (w/v) EDTA in 50% Tris-buffered saline:50% Ca^{2+}-Mg^{2+}-free Dulbecco's Modified Eagle's Medium (DMEM) and treated in suspension with rabbit complement plus anti-GalC monoclonal antibody [to kill oligodendrocytes (Raff *et al.*, 1978)], and A2B5 monoclonal antibody [to kill young oligodendrocytes, type 2 astrocytes and O-2A progenitors (Abney *et al.*, 1983; Raff *et al.*, 1983a, 1983b)]. The remaining cells were plated onto poly-L-lysine coated glass cover-slips at 20 000 or 40 000 cells/cover-slip, irradiated with 2000 R to prevent further cell division, and grown until use in DMEM plus 10% fetal calf serum, 2 mM glutamine and 25 μg/ml gentamycin. These cultures contained 95% $GFAP^+$ cells, which were negative for labelling with A2B5 and with tetanus toxin, and thus had the antigenic phenotype of type 1 astrocytes. Cultures contained no oligodendrocytes, type 2 astrocytes, O-2A progenitors, neurons or macrophages, as determined by labelling with cell-type specific antibodies (see Noble *et al.*, 1984).

Optic nerve cultures

Optic nerves were dissected from 7-day-old rats and prepared for culture as described elsewhere (Raff *et al.*, 1983b). For most experiments, 10 000 dissociated optic nerve cells were plated in 25 μl drops onto PLL-coated cover-slips or directly onto type 1 astrocytes growing in defined medium consisting of DMEM with various additives as described previously (Bottenstein and Sato, 1979; Raff *et al.*, 1983b). After 1–2 h cultures on PLL-glass were fed with 0.5 ml of defined medium or 0.5 ml of defined medium which had been conditioned for 24 h in flasks of confluent type 1 astrocytes. After 1, 2, 3 or 10 days in culture the cells were studied directly on the glass cover-slips by indirect immunofluorescence using 2 fluorochromes.

Immunofluorescence

All antibodies used have been described previously (Raff *et al.*, 1983a, 1983b). In experiments where cells were simultaneously labeled with A2B5 and monoclonal anti-galactocerebroside (GalC), monoclonal antibody A2B5 (Eisenbarth *et al.*, 1979; ascites, 1:1000) was visualized with a class-specific goat anti-mouse IgM antisera conjugated with rhodamine (anti-IgM-Rd; Nordic, 1:40). Monoclonal anti-GalC (Ranscht *et al.*, 1982; ascites, 1:2000) was visualized with a class-specific goat anti-IgG_3 conjugated with fluorescein (anti-IgG_3-Fl; Nordic, 1:80). Cultures were then fixed in methanol (−20°C, 10 min). Alternatively, cultures were labeled sequentially with either A2B5 or anti-GalC and rhodamine-conjugated anti-MIg, fixed in methanol (−20°C, 10 min), and further labeled with anti-GFAP (Pruss, 1979; 1:2000) and fluorescein-conjugated anti-RIg. Double-labelling with A2B5 and anti-GFAP was used to determine that $A2B5^+$ cells were not $GFAP^+$; combined with the information obtained by double-labelling with A2B5 and anti-GalC, these experiments indicated that the $A2B5^+$ $GalC^-$ cells were also $GFAP^-$. After washing, cover-slips were mounted in a drop of Citifluor to prevent the fading of fluorescein (Johnson *et al.*, 1982; Davidson and Goodwin, 1983) and sealed with nail varnish. Alternatively, in some experiments cells were labeled with rabbit antisera against glial fibrillary acidic protein (anti-GFAP and fluorescein-conjugated sheep anti-rabbit IgG (Wellcome, 1:100). Cover-slips were then mounted, sealed and viewed on a Zeiss Universal microscope equipped with phase contrast and epi-u.v. illumination and selective filters for rhodamine and fluorescein, using a x63 Planapo or a x25 Plan-Neofluar objective.

Autoradiography

After optic nerve cultures were established for 18 h, 1 μCi of [^{3}H]thymidine was added to each culture for either 20 h or 6 h. In some experiments [^{3}H]-thymidine was added for a 20-h pulse immediately after the optic nerve cells were plated; these experiments gave comparable results to the experiments where cultures were first grown for 18 h before adding [^{3}H]thymidine. When the radio-labelling period was over, the cover-slips were labeled with antibodies and fluorescent conjugates, dehydrated in ethanol, air-dried, mounted face-up on microscope slides, dipped in autoradiographic emulsion (Ilford, L4) and exposed for 2 days in a light-tight box stored at 4°C. A drop of Citifluor was put onto the cultures and they were then covered with another cover-slip, sealed with nail varnish and examined for radio-labelled and immuno-labelled cells.

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