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A Distinct Type of GD3$^+$, Flat Astrocyte in Rat CNS Cultures

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We have identified what is apparently a distinct type of astrocyte in primary cultures from several regions of the neonatal rat CNS. These cells express GD3 ganglioside for long periods *in vitro*, and are GFAP$^+$, but do not express the oligodendrocyte antigens O4 or galactocerebroside (GC). The majority, but not all, are A2B5$^+$. The cells grow in a flat, highly spread morphology with many thin cytoplasmic processes. Gene transfer with a replication-deficient retrovirus combined with immunostaining for astro- and oligodendroglial markers (antibodies to GFAP, GD3 ganglioside, GC, and the A2B5 and O4 antibodies) demonstrated that in the neonatal rat CNS cultures these cells are clonally separate from oligodendrocytes and from the majority of (GD3$^-$) astrocytes. The clonal analysis suggests a distinct progenitor cell and a distinct developmental sequence for these astrocytes.

The majority of astrocytes and oligodendrocytes are generated in the mammalian CNS during the latter stages of gestation and early postnatal period. The idea that separate precursor cells generate these two glial cell classes is supported by a large number of studies *in vivo* (Curtis et al., 1988; LeVine and Goldman, 1988; Luskin et al., 1988; Price and Thurlow, 1988; Reynolds and Wilkin, 1988; Skoff, 1990). Although observations of morphology, immunocytochemical characteristics, and receptor expression in glial cultures have noted variations in shape and antigen expression within each of these glial types (see, e.g., Raff et al., 1983a; Behar et al., 1988; Lerea and McCarthy, 1989), the question of diversity has generally not been addressed in lineage studies. That is, are there distinct progenitors for different varieties of astrocytes and oligodendrocytes? In primary cultures of rodent CNS, two major progenitor populations have been described (Raff et al., 1983b; Aloisi et al., 1988; Behar et al., 1988; Luskin et al., 1988; Ingraham and McCarthy, 1989; Raff, 1989). One generates astrocytes characterized by a broad, flat morphology and by the absence of A2B5 antibody binding (type 1 astrocytes). The other (O-2A progenitors) generates oligodendrocytes or stellate, A2B5$^+$ astrocytes (type 2 astrocytes). Thus, different progenitors can generate two different astrocyte types, at least in culture.

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We have recently pursued the question of glial cell lineages in cultures from neonatal rat CNS using a replication-deficient retrovirus and triple immunofluorescence labeling (Vaysse and Goldman, 1990). Immunological markers included a monoclonal antibody to GD3 ganglioside, a major glycolipid component of immature neuroectodermal cells and immature oligodendrocytes (Goldman et al., 1984; LeVine and Goldman, 1988; Reynolds and Wilkin, 1988); O4, a monoclonal antibody that binds to developing oligodendrocytes (Sommer and Schachner, 1981; Gard and Pfeiffer, 1989); and antibodies to galactocerebroside (GC), for oligodendrocytes (Raff et al., 1978), and glial fibrillary acidic protein (GFAP), for the identification of astrocytes (Bignami et al., 1972). The results indicated separate lineages for oligodendrocytes and astrocytes. During the course of these clonal studies, we found a type of astrocyte that appeared to be morphologically, antigenically, and clonally separate both from type 1 astrocytes and from oligodendrocytes/type 2 astrocytes. These cells are large, display a flat morphology, often with thin, curving, cytoplasmic processes, and are GFAP$^+$/GD3$^+$ even after 2 months *in vitro*. In this report, we present further characterization of these cells and provide evidence that they are derived from a proliferating cell population separate from the O-2A progenitor, which gives rise to oligodendrocytes/type 2 astrocytes, and from the progenitor population that gives rise to the type 1 astrocytes.

Materials and Methods

Cell cultures. Primary cultures were established from newborn and from embryonic day 16 (E16) Sprague–Dawley rat CNS. Brains and spinal cords were removed aseptically and placed in ice-cold Dulbecco's phosphate-buffered saline (PBS), pH 7.4. Cultures were established and maintained as either explants or dissociated cultures. Explant cultures were prepared from fragments of striata, which included the subventricular zone (SVZ) at the angle of the lateral ventricle, or fragments of the lumbosacral spinal cord. The areas of interest were rapidly dissected in ice-cold PBS and minced into small pieces (about 0.3–0.5 mm) for growth as explants. Two to five pieces were seeded onto 12 mm round glass coverslips coated with poly-L-lysine (10 μg/ml), and placed in 24-well tissue culture plates (Falcon Labware, Thomas, NJ). Cultures were maintained in 1.0 ml of Waymouth's MD 705/1 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) for up to 8 weeks, with no change of medium. This method allows the growth of astrocytes and oligodendrocytes in large numbers (Vaysse and Goldman, 1990). Waymouth MD 705/1 medium has been reported to provide favorable conditions for the growth of oligodendrocytes in culture (Roussel et al., 1981; Espinosa de los Monteros et al., 1986).

For dissociated cultures, tissue from the SVZ was minced and incubated with 0.05% trypsin and 0.53 mM EDTA (GIBCO, Grand Island, NY) for 15 min at 37°C in a shaker. Cells were dissociated by several passages through a Pasteur pipette, the trypsin was inactivated with serum, and a single-cell suspension was seeded onto coverslips at 2.5×10^4 cells/cm^2. Cultures were maintained in a minimum essential medium (MEM)-based medium containing 10% fetal bovine serum (Goldman and Chiu, 1984), with medium changed twice a week. In

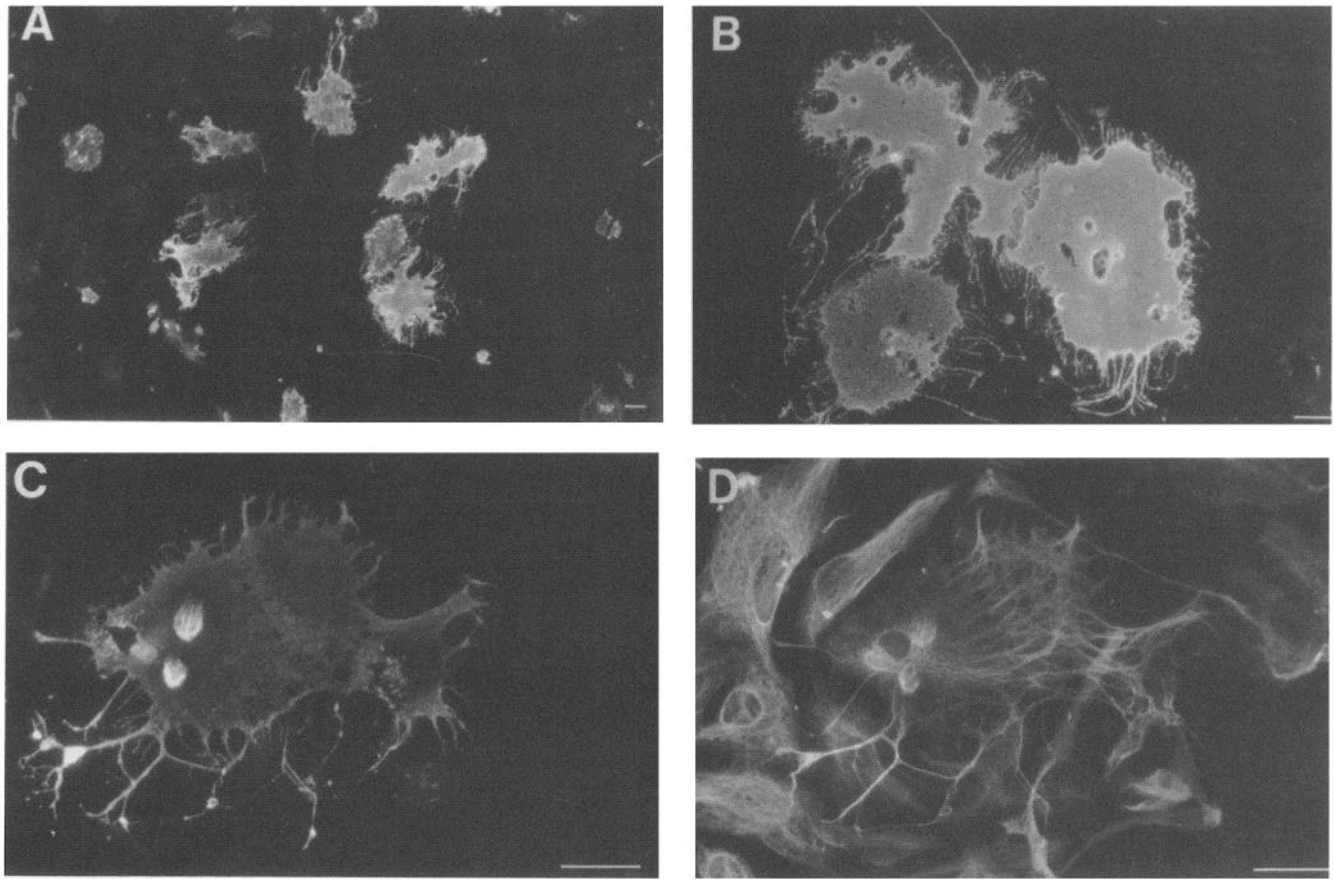

Figure 1. Morphology of GD3$^+$ astrocytes (visualized with IgG_3-RITC). *A,* Low-power photomicrograph of field containing flat, GD3$^+$ astrocytes. Immunostaining of the same field showed that the GD3$^+$ cells were also GFAP$^+$, as were many GD3$^-$ cells in the field (not shown). *B,* Higher magnification of a cluster of three GD3$^+$ astrocytes that displayed characteristic thin cytoplasmic extensions. *C* and *D,* Anti-GD3 (rhodamine) staining (*C*) and anti-GFAP (fluorescein) staining (*D*) of the same field showing that the GD3$^+$, flat cell is also GFAP$^+$. Note several polygonal GD3$^-$/GFAP$^+$ cells in the same field. Scale bars, 50 μm.

contrast to the first method, this protocol largely produces astrocytes, with few oligodendrocytes.

All cultures were maintained at 37°C in humidified 95% air, 5% CO_2. All sera and media solutions were purchased from GIBCO (Grand Island, NY).

Infection with the BAG retrovirus. The construction and production of the BAG recombinant retrovirus are described in detail by Price et al. (1987), and its use in neonatal cultures from rat CNS, by Vaysse and Goldman (1990). After various days *in vitro* (DIV), cultures were infected with a 5 μl dose of the BAG retrovirus stock suspension. We previously established that this dose produced a small number of infections (no more than 10 clusters/cm^2). A small clonal number is important in order to avoid overlapping of neighboring clones. Coverslips were removed for β-galactosidase and immunofluorescence staining at various times after the virus infection.

X-gal staining and indirect immunofluorescence. Histochemical staining for β-galactosidase (X-gal staining) and indirect immunofluorescence were performed as previously described (Vaysse and Goldman, 1990).

Antibodies. We used a rabbit polyclonal anti-GFAP antiserum (Goldman and Chiu, 1984) as an astrocyte marker, a rabbit polyclonal anti-GC antiserum (Norton et al., 1983) as an oligodendrocyte marker, and mouse monoclonal antibodies R24 (Pukel et al., 1980), A2B5 (Eisenbarth et al., 1979; Raff et al., 1983a), and O4 (Sommer and Schachner, 1981). The secondary antibodies used included rhodamine isothiocyanate (RITC)-conjugated goat IgG specific for mouse IgG_3 (IgG_3-RITC; Southern Biotechnology, Birmingham, AL), fluorescein isothiocyanate (FITC)-conjugated goat IgG specific for mouse IgM (IgM-FITC; Southern Biotechnology, Birmingham, AL), and 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated goat IgG raised against rabbit IgG (IgG-AMCA; Jackson ImmunoResearch, West Grove, PA). Controls for specificity were performed as previously described (Vaysse and Goldman, 1990).

Results

Morphology and antigen expression

The astrocytes described in this report were originally seen in both striatal and spinal cord cultures from neonatal rats (Vaysse and Goldman, 1990). They bound the monoclonal antibody R24, an anti-GD3 ganglioside antibody, and thus were easily distinguished from the large majority of the astrocytes, which were GD3$^-$/GFAP$^+$. They typically spread extensively on the substrate and displayed many fine, cytoplasmic processes (Fig. 1). Some of these processes appeared as short, spikelike projections, while others were long and curving (up to 90 μm). The nuclei of these cells were oval (approximately 15 μm × 10 μm), similar to those of the GD3$^-$ astrocytes, but unlike the typical round and smaller, eccentrically placed nuclei of oligodendrocytes and type 2 astrocytes. The GD3$^+$, flat astrocytes comprised a small proportion of the total cells in these cultures (approximately 0.6–0.9% after 2 weeks) and grew as isolated cells or in small clusters (Fig. 1*A,B*).

The morphological and antigenic phenotypes of these cells distinguished them from type 1 astrocytes and from oligodendrocytes and oligodendrocyte precursors (Table 1, Fig. 2). Type 1 astrocytes were GD3$^-$/O4$^-$/GFAP$^+$ and assumed a polygonal

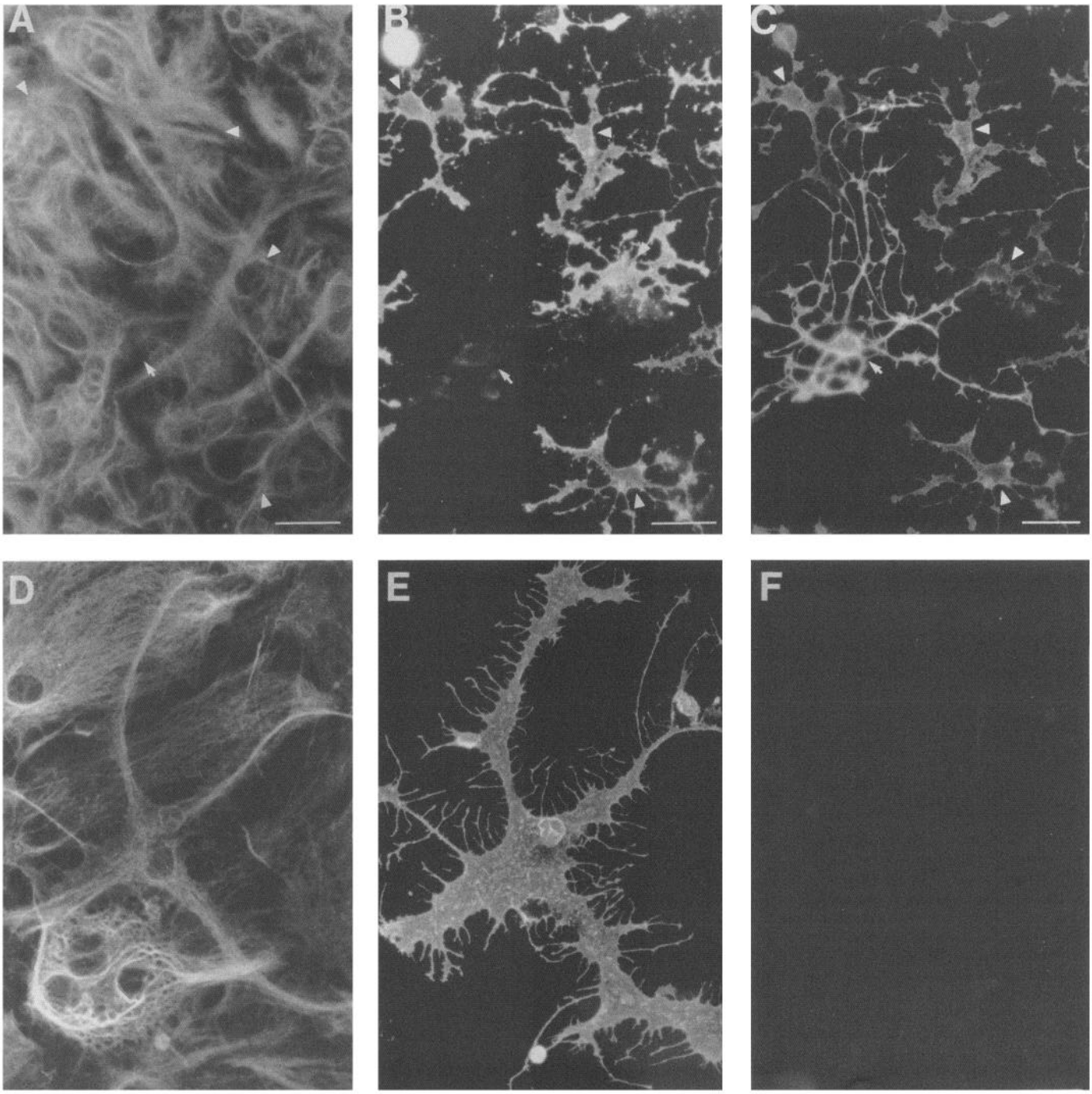

Figure 2. Illustration of the phenotypic characteristics of the GFAP$^+$/GD3$^+$, flat astrocytes by triple-label indirect immunofluorescence for GFAP, GD3, and O4. Each *panel* in each *triplet* (*A–C* and *D–F*) represents a single label of a triple-stained field. *A* and *D*, anti-GFAP antibody (AMCA); *B* and *E*, anti-GD3 antibody (rhodamine); *C* and *F*, O4 antibody (fluorescein). *A–C* show many GFAP$^-$/GD3$^+$/O4$^+$, process-bearing cells of the oligodendrocyte lineage (*arrowheads*), and one GFAP$^-$/GD3$^-$/O4$^+$ oligodendrocytic cell (*B, arrow*). The GFAP$^+$ cells in this field are GD3$^-$/O4$^-$ (type 1 astrocytes). *Arrowheads* in *A* show the positions of the GD3$^+$ and O4$^+$ cells relative to the astrocytes upon which they sit. *D–F* show a GFAP$^+$/GD3$^+$/O4$^-$ cell growing with GFAP$^+$/GD3$^-$/O4$^-$ cells. Scale bars, 50 μm.

shape (Fig. 2). Oligodendrocytes and their precursors were GFAP$^-$ and showed a process-bearing morphology (Fig. 2*A–C*). Under the culture conditions used, >90% of cells in the oligodendrocyte lineage became O4$^+$ after 3–4 weeks (Vaysse and Goldman, 1990). The unusual astrocytes described here were always flat, GD3$^+$, and GFAP$^+$ (Figs. 1, 2*D–F*, 3, 4), but never expressed either O4 or GC, even after 6 weeks in culture, and did not display the multibranched, process-bearing morphology of oligodendrocytic cells. Many, but not all, of the GD3$^+$, flat astrocytes also bound the A2B5 antibody (e.g., 56 of 100 randomly picked GFAP$^+$/GD3$^+$, flat cells in one culture 43 DIV were also A2B5$^+$) (Fig. 3).

The morphological and antigenic features of these cells appeared to be maintained for 6–8 weeks (the longest time ex-

amined), since we could find such cells in the older cultures, in which they continued to comprise about 1% of the total cells. Since there is little or no cell proliferation in these cultures after 2 weeks (Vaysse and Goldman, 1990), the continued presence of the GD3$^+$, flat astrocytes at a constant proportion of the total cell number suggests that they represent a stable, nonproliferating population that maintains its phenotype and does not convert to GD3$^-$ astrocytes.

We examined cultures maintained in two different media for these astrocytes. In the first, small explants or fragments of tissue were grown in Waymouth's medium plus 10% FCS, a condition in which both astrocytes and large numbers of oligodendrocytes develop. In the second, dissociated tissue was grown at low density (3000 cells/cm^2 at 3 DIV) in an MEM-based medium containing 10% FCS, a condition in which few oligodendrocytes develop and the cultures are composed almost entirely of GD3$^-$ astrocytes. The GD3$^+$, flat astrocytes were seen in both growth conditions and indeed showed identical morphological and antigenic characteristics.

We also established cultures from E16 rat forebrains. Precursors for the flat, GD3$^+$ astrocytes were present at this age and were able to generate the cells, since we were able to recognize these cells by morphology and antigen expression by 4 DIV. As in the cultures grown from postnatal day 0 (P0) rats, the GD3$^+$, flat astrocytes comprised a small proportion of the total cell population (0.5–1%).

Clonal analysis

To determine whether the GD3$^+$, flat astrocytes were derived from the same or from a different proliferative cell population as those of the majority of astrocytes and of oligodendrocytes, we infected P0 cultures from 1 to 3 DIV with the BAG cell vector and examined them between 2 and 4 weeks *in vitro*. Cultures were first stained with X-gal to visualize clones and then by indirect immunofluorescence with anti-GD3, O4, and anti-GFAP antibodies. Under both culture conditions (see above) the GD3$^+$, flat astrocytes appeared clonally distinct from other cell types (Table 2).

In dissociated cultures from the SVZ region grown in MEM with 10% FCS, we found two types of astrocytes, the large majority (approximately 99%) being GD3$^-$/O4$^-$/GFAP$^+$, and a small minority being GD3$^+$/O4$^-$/GFAP$^+$. No oligodendrocytes were observed (O4$^+$ or GC$^+$ cells). Two types of clones were found, corresponding to the two types of astrocytes. The large majority of the clones (approximately 99%) contained exclusively GD3$^-$/O4$^-$/GFAP$^+$ cells, with clonal sizes varying from 5 to more than 100 cells. A second type of clone contained exclusively GD3$^+$/O4$^-$/GFAP$^+$, flat cells. Four of these were observed, containing, respectively, two, two, four, and six cells (Fig. 4). No clones containing both GD3$^+$ and GD3$^-$ astrocytes were found.

In explant cultures from the SVZ region or spinal cord grown in Waymouth medium with 10% FCS, we found three types of clones. The distribution of clones reflected closely the proportions of the various cell types in the cultures. Approximately half of the clones contained exclusively GD3$^-$/O4$^-$/GFAP$^+$ cells, with clonal sizes varying from 2 to more than 100 cells. Other clones contained mixtures of GD3$^+$, O4$^+$, process-bearing cells, with clonal sizes varying from 2 to more than 100 cells. These clones represent cells in the oligodendrocyte lineage (Vaysse and Goldman, 1990). Importantly, the GD3$^+$, flat astrocytes were excluded from either of these two types of clones (approximately 99% of the total number of clones). No clones containing both GD3$^+$ and GD3$^-$ astrocytes were found, and none contained both GD3$^+$, flat astrocytes and process-bearing cells. Whenever we found a GD3$^+$, flat astrocyte in a clone, all the cells present in that clone had the same phenotype. Four such clones, containing, respectively, one, two, two, and eight GD3$^+$/O4$^-$/GFAP$^+$, flat cells were found.

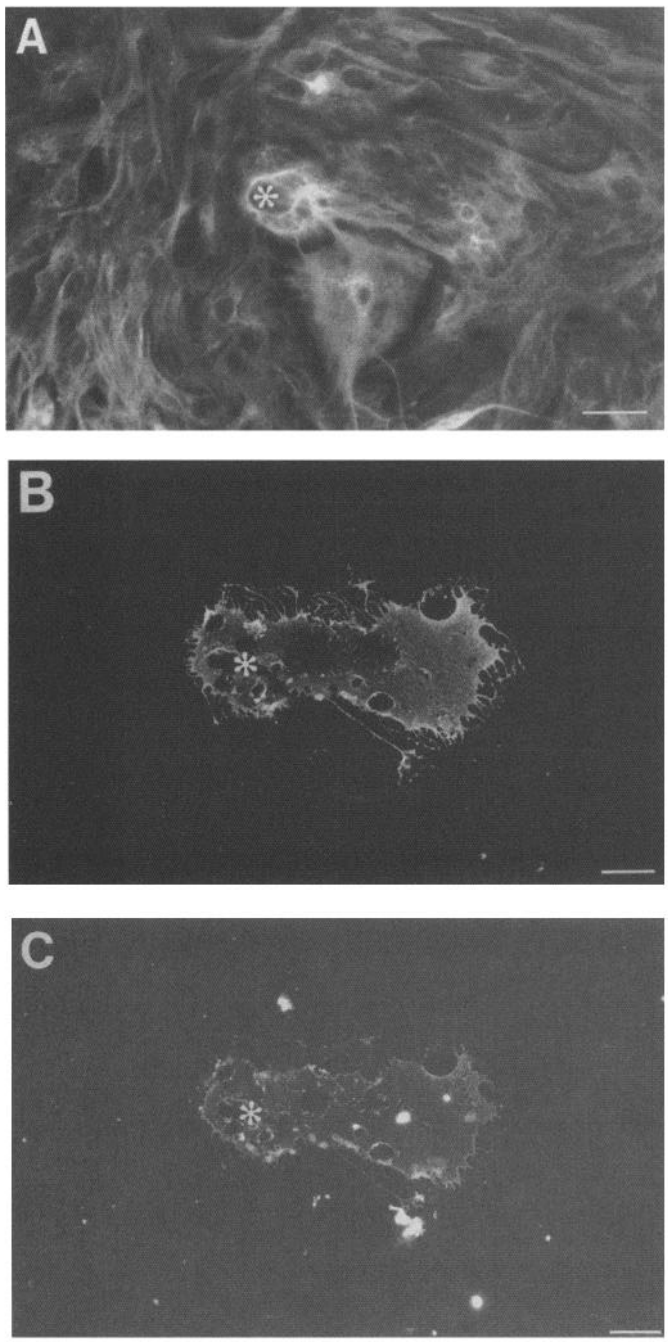

Figure 3. Triple-label immunofluorescence with anti-GFAP antibody (AMCA; *A*), anti-GD3 antibody (rhodamine; *B*) and A2B5 antibody (fluorescein; *C*). An *asterisk* marks the position of the GFAP$^+$/GD3$^+$/A2B5$^+$ cell. Scale bars, 50 μm.

To determine whether the GD3$^+$, flat astrocytes could be generated from embryonic CNS, we infected E16 explant cultures with the BAG virus after 1 DIV and analyzed them between 6 and 13 d later. Two types of clones were observed. Of 23 clones

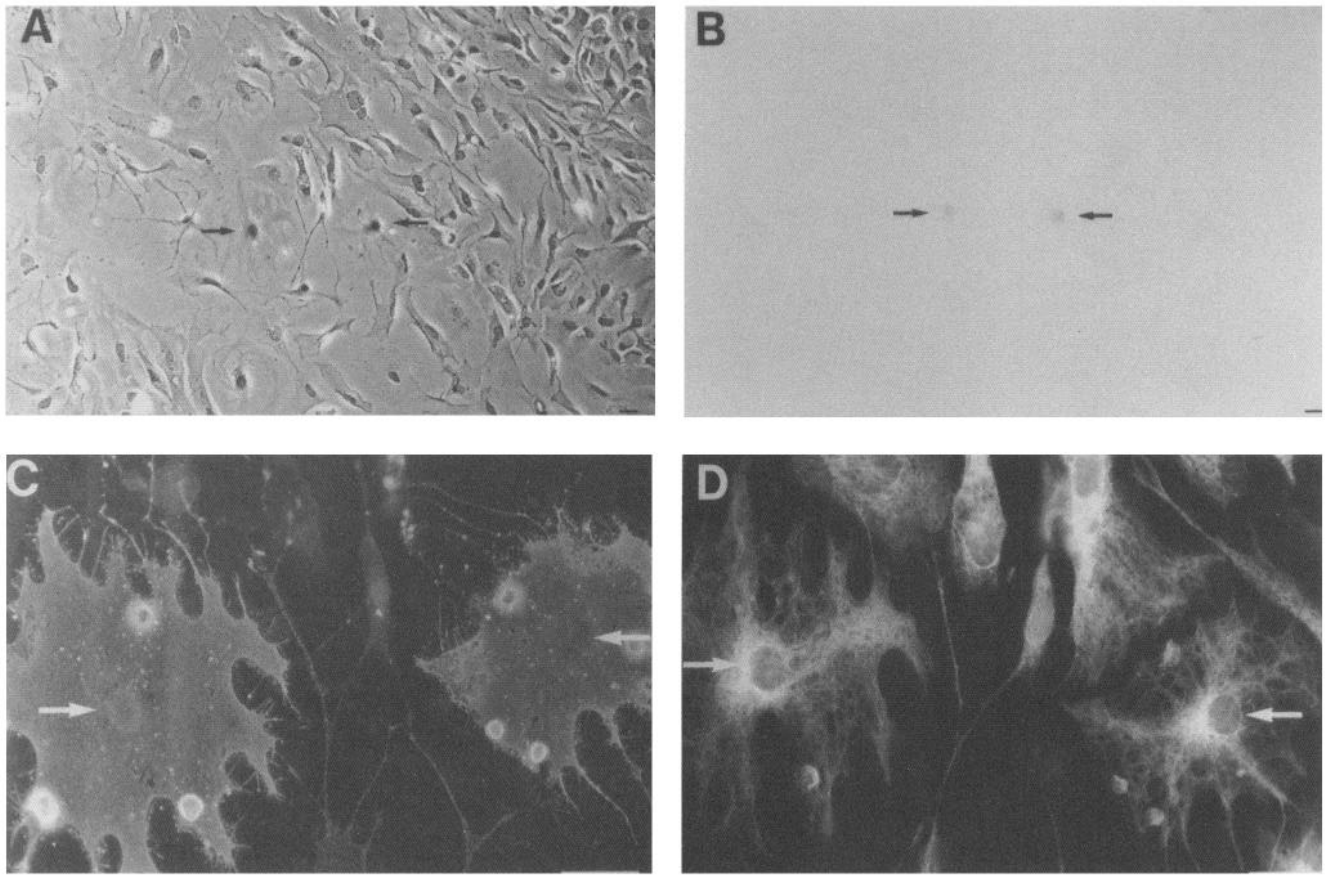

Figure 4. A clone of two GFAP⁺/GD3⁺ flat cells (*arrows*). *A,* Phase contrast at low magnification. All other cells in the field were GD3⁻/GFAP⁺. *B,* X-gal staining. *C,* Anti-GD3 antibody (rhodamine). *D,* Anti-GFAP antibody (fluorescein). Scale bars, 50 μm.

at 7 DIV, 19 were composed solely of GFAP⁺/GD3⁻ cells (type 1 astrocytic) and 4 were composed solely of GFAP⁻/GD3⁺ process-bearing cells (oligodendrocytic). Of 34 clones at 14 DIV, 29 were only type 1 astrocytic and 5 were only oligodendrocytic. No clones of the GD3⁺, flat astrocytes were observed, and none of these cells was found as part of any of the 57 clones, even though the cells were present in the cultures (see above).

Because of the probability that precursors for the flat, GD3⁺ cells make up only a small part of the mitotically native cells in late embryonic life or indeed are not mitotically active at all at the time of infection, we infected four coverslips of E16 cultures of forebrain with a higher dose of virus (10-fold higher) at 1 DIV and analyzed cultures at 12 DIV. A large number of X-gal–positive cells were observed, too many to allow a clonal

Table 1. Morphological features and phenotypes of the various glial cell types found in primary explant cultures of the neonatal rat CNS

		Antigenic phenotype				
Cell type	Morphology	GFAP	A2B5	GD3	O4	GC
Astrocytes (GFAP⁺)						
Type 1 astrocyte	Flat, polygonal	+	−	−	−	−
Type 2 astrocyte	Multipolar	+	+	±	−	−
GD3⁺, flat astrocyte	Flat, with thin processes	+	±	+	−	−
Oligodendrocyte precursors and oligodendrocytes (GC⁺)						
O-2A progenitor	Bipolar	−	+	+	−	−
O4⁺ progenitor	Multipolar	−	±	±	+	−
Oligodendrocyte	Multipolar	−	−	−	+	+

Explant cultures from neonatal rat striatum or spinal cord were grown in Waymouth's medium with 10% FCS, fixed between 14 and 28 DIV, and labeled with a combination of three of four antibodies (anti-GD3, O4, anti-GFAP, anti-GC, A2B5). Morphology and antigen expression of the GD3⁺, flat astrocytes are noted in comparison to those of other astrocyte and oligodendrocyte types seen in the cultures established for these experiments and also described in many previous studies of rodent CNS *in vitro* (see, e.g., Raff et al., 1983a, b; Aloisi et al., 1988; Behar et al., 1988; Gard and Pfeiffer, 1989).

analysis because clusters of cells were close together or even overlapped. Nevertheless, 1132 X-gal–positive cells were observed, both type 1 astrocytes and process-bearing oligodendrocytes. Despite the presence of the flat, GFAP$^+$/GD3$^+$ cells in the cultures (0.6% of the total cell population), no such cells expressed β-galactosidase, indicating that these cells, or their precursors, showed little or no proliferation at the time of infection.

Discussion

The flat, GD3$^+$ astrocytes are distinct from type 1 astrocytes and from oligodendrocytes

We have described a type of astrocyte in rat CNS cultures with several characteristic features: these cells express GD3 ganglioside for long periods *in vitro*; many also bind A2B5; they do not express either of the oligodendrocyte antigens, O4 and GC; they display a highly spread and irregular morphology often with many thin processes; and they do not proliferate well under the culture conditions used.

The GD3$^+$, flat astrocytes were observed in cultures grown under two different conditions. In Waymouth's medium, both astrocytes and oligodendrocytes develop (Vaysse and Goldman, 1990), while dissociated cell cultures maintained at low density in MEM with FCS consist of astrocytes. Apparently, the flat, GD3$^+$ astrocytes, like the GD3$^-$ astrocytes, are not as sensitive to culture conditions as cells of the oligodendrocyte lineage (Raff et al., 1983b). These GD3$^+$, flat astrocytes comprised a small proportion of the total cells in these cultures (approximately 0.6–0.9% after 2 weeks). These numbers are consistent with the observations that the clonal size is small and that they grow as isolated cells or in small clusters. Their proliferative capacity *in vitro* thus appears to be far less than that of oligodendrocytic cells or GD3$^-$ astrocytes. It is therefore likely that at the time the cultures are first established, the proportion of the GD3$^+$ astrocyte type, or its progenitor, is far larger than 0.6–0.9%. As an example, in one experiment the total numbers of cells on each coverslip increased from 5×10^3 at 5 DIV to 1×10^5 after 20 DIV. At 20 DIV there were about 500 GD3$^+$, flat astrocytes (0.5%). If these cells divided on average once or twice, as indicated by clonal size, then they would have represented about 2.5–5% of the total at 5 d. We cannot yet directly count the numbers of these cells in early cultures for several reasons. First, the characteristic flat morphology with thin processes takes several days to develop. Second, in explants, it takes at least 2 d for cells to begin migrating from the tissue. Third, in the first few days of forebrain cultures, there are many process-bearing GD3$^+$ cells (Goldman et al., 1986). Some may lose GD3 expression, while a small percentage may retain it. Thus, the morphology and antigen expression of the precursor for the GD3$^+$, flat astrocytes remain undefined (see below).

One complication in defining these GD3$^+$, flat astrocytes is that there is not an exact correlation between morphology and ganglioside expression. For example, while type 2 astrocytes are typically process-bearing cells, several reports suggest that type 2 astrocytes maintained in culture for long periods may flatten out and even lose GD3 and A2B5 (Raff et al., 1983a; Goldman et al., 1986; Aloisi et al., 1988). GD3$^+$ or A2B5$^+$, flat astrocytes have been noted in cultures from optic nerve, forebrain, and cerebellum (Raff et al., 1983a; Johnstone et al., 1986; Aloisi et al., 1988; Behar et al., 1988). It has not been clear whether they should be defined as type 1 or type 2 astrocytes. It is possible that some of these previously noted flat, A2B5$^+$, GD3$^+$ astrocytes are those we describe.

Table 2. Phenotypes of clones in cultures of neonatal SVZ and spinal cord *in vitro*

Culture technique/ medium	SVZ		Spinal cord:
	Dissociated MEM	Explants Waymouth	Explants/ Waymouth
% GD3$^+$/GFAP$^+$ cells	0.7%	0.9%	2.2%
Total clones	376	374	563
Flat, GD3$^-$/GFAP$^+$ cell clones	372	210	291
Process-bearing cell clones	0	163	269
Flat, GD3$^+$/GFAP$^+$ cell clones	4	1	3

Cultures were infected with 5 μl of retrovirus stock supernatant from 1 to 3 DIV, fixed between 14 and 28 DIV, and quadruple labeled with X-gal plus a combination of anti-GD3, O4, and anti-GFAP antibodies. The process-bearing cell clones contained mixtures of GD3$^+$/O4$^-$, GD3$^+$/O4$^+$, and GD3$^-$/O4$^+$ cells, but no GFAP$^+$ cells.

The clonal analysis indicates that the GD3$^+$, flat astrocytes are derived from a pool of precursors distinct from those of oligodendrocytes and of the other (GD3$^-$) astrocytes. In studying a large number of clones in the present and in a previous study (Vaysse and Goldman, 1990), these GD3$^+$, flat astrocytes were never found to be part of oligodendrocytic or GD3$^-$ astrocytic clones. Furthermore, clones of GD3$^+$, flat astrocytes contained only similar cells. This separation of GD3$^-$ and GD3$^+$ astrocytes between clones is extraordinarily unlikely to be generated by random assortment. We estimate that the probability of obtaining 4 clones containing exclusively 14 GD3$^+$ astrocytes and 372 clones containing exclusively 1992 GD3$^-$ astrocytes (see Table 2) would be less than 1 in 10^{30} if cells were randomly assorted among the 376 clones, that is, if the two astrocyte types had a common precursor that gave rise to clones containing both types of cells, one type representing 99% of the astrocytes and the other 1% of astrocytes. We therefore conclude that the population of precursors that give rise to the GD3$^-$ astrocytes is different from the one that generates the GD3$^+$, flat astrocytes. This does not necessarily mean that the precursors for the type 1 astrocytes and the GD3$^+$, flat astrocytes are unrelated or that they belong to different developmental sequences (see below).

We also infer that the progenitor of the GD3$^+$ astrocytes described in this report and the progenitor of oligodendrocytes are distinct from each other at the time of retroviral infection (1–3 DIV from cultures of P0 rat forebrain and cord). Although the antigen expression of the GD3$^+$, flat astrocytes (GFAP$^+$/GD3$^+$/±A2B5$^+$) is the same as that of astrocytes of the O-2A lineage, the type 2 astrocytes (Raff et al., 1983a), we do not believe these cells represent type 2 astrocytes. This conclusion is drawn from the observation that the GD3$^+$ astrocytes were never seen as members of oligodendrocytic clones in conditions in which both astrocytes and oligodendrocytes develop. Indeed, the occasional GFAP$^+$ cell in oligodendrocytic clones displayed a process-bearing, not a spread morphology (Vaysse and Goldman, 1990).

While the clonal analysis shows that the precursors for type 1 astrocytes, oligodendrocytes/type 2 astrocytes, and the GD3$^+$, flat astrocytes are distinct from one another at the time of viral infection, the relationships among these different precursors and the nature of the precursors are not clear. One possibility is that the three types of clones indeed represent three distinct lineages,

with common precursors at some earlier point. Another possibility is that the GD3$^+$, flat astrocytes are derived from part of a developmental sequence that eventually gives rise to the type 1 astrocytes or to an oligodendrocyte sequence. The neonatal CNS at any one time undoubtedly contains immature glia in different stages of development. If a developmental sequence is altered by removing cells from the CNS and placing them in culture, then it is possible that precursors at an earlier stage in the sequence could develop in a different or aberrant manner and thus appear clonally distinct from more mature cells in the same lineage. Our observations in the E16 cultures suggest that this hypothesis is not the case. Progenitors for type 1 astrocytes and for oligodendrocytes are dividing and appear to be distinct at E17, the time of infection. Cells of the flat, GD3$^+$ type were indeed observed in these cultures, indicating that their progenitor(s) are present at that time and able to develop into the cells. However, no BAG virus–infected cells were observed, indicating little or no proliferation of these cells or their precursors at that time. Further studies on the nature and fates of the progenitors present in early cultures are required to answer those questions.

Are the GD3$^+$, flat astrocytes present in many regions of the CNS?

We found the GD3$^+$, flat astrocytes as clonally distinct populations in cultures from both forebrain and spinal cord. We have examined cultures of neonatal optic nerve and cerebellum and found morphologically and antigenically similar astrocytes after 1–3 weeks *in vitro*. Without further analysis, however, we cannot determine whether these cells are similar in their properties to those in other regions and clonally distinct from other astrocytes and from oligodendrocytes.

Do the GD3$^+$, flat astrocytes correspond to glial forms in vivo*?*

Correlations between what one observes in tissue culture and the various types of glial cells present in the CNS is a critical question for interpreting any studies of glial cell structure, function, or lineage *in vitro*. In recent immunocytochemical studies of developing rat forebrain and cerebellum, cells expressing both GD3 ganglioside and GFAP were sought but were not found (Curtis et al., 1988; LeVine and Goldman, 1988; Reynolds and Wilkin, 1988). In rat spinal cord, however, radial glial forms express GD3 until the end of gestation and into the first few postnatal days, and it is possible to find GD3$^+$/GFAP$^+$ cells (Hirano and Goldman, 1988). The GD3 antibody binding does not persist for more than a few days, however.

The only known long-term expression of GD3 ganglioside by astrocytes is in CNS scar tissues. GD3 is typically elevated in a variety of pathological disorders, including such human neuropathological conditions as multiple sclerosis and subacute sclerosing panencephalitis (Ledeen et al., 1968; Yu et al., 1974) and such murine cerebellar mutants as *lurcher, staggerer,* and *Purkinje cell degeneration* (Seyfried and Yu, 1984). GD3 ganglioside has been localized to astrocytes in the cerebella of these mutants (LeVine et al., 1986) and to astrocytes in human CNS scars (J. E. Goldman, unpublished observations). Interestingly, the astrocytes in murine cerebellar mutants display an extraordinary growth of thin cytoplasmic sheets and processes, some of which wrap around residual mossy fiber terminals (Hirano and Dembitzer, 1976; Mullen et al., 1976; Ghetti et al., 1981; LeVine et al., 1986). Whether GD3 expression in astrocytes represents an induction during pathological reactions or the presence of an already existing GD3$^+$ astrocyte population remains to be determined. A further comparison between the GD3$^+$ astrocytes of gliotic tissues and the GD3$^+$ astrocytes observed in cultures would be required before drawing any conclusions about similarities or differences.

References

Aloisi F, Agresti C, Levi G (1988) Establishment, characterization, and evolution of cultures enriched in type 2 astrocytes. J Neurosci Res 21:188–198.

Behar T, McMorris FA, Novotny EA, Barker JL, Dubois-Dalcq M (1988) Growth and differentiation properties of O-2A progenitors purified from rat cerebral hemispheres. J Neurosci Res 21:168–180.

Bignami A, Eng L, Dahl D, Uyeda C (1972) Localization of the glial fibrillary acidic protein in the astrocytes by immunofluorescence. Brain Res 43:429–431.

Curtis R, Cohen J, Fok-seang J, Hanley MR, Gregson NA, Reynolds R, Wilkin GP (1988) Development of macroglial cells in rat cerebellum. I. Use of antibodies to follow early *in vivo* development and migration of oligodendrocytes. J Neurocytol 17:43–54.

Eisenbarth GS, Walsh FS, Nirenberg M (1979) Monoclonal antibody to a plasma membrane antigen of neurons. Proc Natl Acad Sci USA 76:4913–4917.

Espinosa do los Monteros A, Roussel G, Nussbaum JL (1986) A procedure for long-term culture of oligodendrocytes. Dev Brain Res 24:117–125.

Gard AL, Pfeiffer SE (1989) Oligodendrocyte progenitors isolated directly from developing telencephalon at a specific phenotypic stage: myelinogenic potential in a defined environment. Development 106: 119–132.

Ghetti B, Truex L, Sawyer B, Strada S, Schmidt M (1981) Exaggerated cyclic AMP accumulation and glial cell reaction in the cerebellum during Purkinje cell degeneration in pcd mutant mice. J Neurosci Res 6:789–801.

Goldman JE, Chiu FC (1984) Growth kinetics, cell shape, and the cytoskeleton of primary astrocyte cultures. J Neurochem 42:175–184.

Goldman JE, Hirano M, Yu RK, Seyfried TN (1984) GD3 ganglioside is a glycolipid characteristic of immature neuroectodermal cells. J Neuroimmunol 7:179–192.

Goldman JE, Geier SS, Hirano M (1986) Differentiation of astrocytes and oligodendrocytes from germinal matrix cells in primary culture. J Neurosci 6:52–60.

Hirano A, Dembitzer HM (1976) The fine structure of astrocytes in the adult staggerer. J Neuropathol Exp Neurol 35:63–74.

Hirano M, Goldman JE (1988) Gliogenesis in rat spinal cord: evidence for origin of astrocytes and oligodendrocytes from radial precursors. J Neurosci Res 21:155–167.

Ingraham CA, McCarthy KD (1989) Plasticity of process-bearing glial cell cultures from neonatal rat cerebral cortical tissue. J Neurosci 9: 63–69.

Johnstone S, Levi G, Wilkin GP, Scheinder A, Ciotti MT (1986) Subpopulation of rat cerebellar astrocytes in primary culture: morphology, cell surface antigens and [^{3}H] GABA transport. Dev Brain Res 24:63–75.

Ledeen R, Salsman K, Cabrera M (1968) Gangliosides in subacute sclerosing leukoencephalitis—isolation and fatty acid composition of nine fractions. J Lipid Res 9:129–136.

Lerea LS, McCarthy KD (1989) Astroglial cells *in vitro* are heterogeneous with respect to expression of the alpha$_1$-adrenergic receptor. Glia 2:135–147.

LeVine SL, Goldman JE (1988) Embryonic divergence of oligodendrocyte and astrocyte lineages in developing rat cerebrum. J Neurosci 8:3992–4006.

LeVine SL, Seyfried TN, Yu RK, Goldman JE (1986) Immunocytochemical localization of GD3 ganglioside to astrocytes in murine cerebellar mutants. Brain Res 374:260–269.

Luskin MB, Pearlman AL, Sanes JR (1988) Cell lineage in the cerebral cortex of the mouse studied *in vivo* and *in vitro* with a recombinant retrovirus. Neuron 1:635–647.

Mullen RJ, Eicher EM, Sidman RL (1976) Purkinje cell degeneration, a new neurological mutation in the mouse. Proc Natl Acad Sci USA 73:208–212.

Norton WT, Farooq M, Fields KL, Raine CS (1983) The long term culture of bulk-isolated bovine oligodendroglia from adult brain. Brain Res 270:295–310.

Price J, Thurlow L (1988) Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. Development 104:473–482.

Price J, Turner D, Cepko C (1987) Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc Natl Acad Sci USA 84:156–160.

Pukel CS, Lloyd KO, Travassos LR, Dippold WG, Oettgen HF, Old LJ (1982) GD3, a prominent ganglioside of human melanoma. J Exp Med 155:1133–1147.

Raff MC (1989) Glial cell diversification in the rat optic nerve. Science 243:1450–1455.

Raff MC, Mirsky R, Fields KL, Lisak RP, Dorman SH, Silberberg DH, Gregson NA, Liebowitz S, Kennedy M (1978) Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture. Nature 274:813–816.

Raff MC, Abney E, Cohen J, Lindsay R, Noble M (1983a) Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. J Neurosci 3:1289–1300.

Raff MC, Miller RH, Noble M (1983b) A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. Nature 303:390–396.

Reynolds R, Wilkin GP (1988) Development of macroglial cells in rat cerebellum II. Development 102:409–425.

Roussel G, Labourdette G, Nussbaum JL (1981) Characterization of oligodendrocytes in primary cultures from brain hemispheres of newborn rats. Dev Biol 81:372–378.

Seyfried TN, Yu RK (1984) Cellular localization of gangliosides in the mouse cerebellum: analysis using neurological mutants. Adv Exp Med Biol 174:169–181.

Skoff RP (1990) Gliogenesis in rat optic nerve: astrocytes are generated in a single wave before oligodendrocytes. Dev Biol 139:149–168.

Sommer I, Schachner M (1981) Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytochemical study in the central nervous system. Dev Biol 83:311–327.

Vaysse PJ-J, Goldman JE (1990) A clonal analysis of glial lineages in neonatal forebrain development *in vitro*. Neuron 5:227–235.

Yu RK, Ledeen RW, Engl LF (1974) Ganglioside abnormalities in multiple sclerosis. J Neurochem 23:169–174.