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Bipotential precursors of putative fibrous astrocytes and oligodendrocytes in rat cerebellar cultures express distinct surface features and "neuron-like" γ-aminobutyric acid transport

(glial differentiation/neurotransmitter amino acids/neural cell immunocytochemistry/cell lineage)

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ABSTRACT **When postnatal rat cerebellar cells were cultured in a chemically defined, serum-free medium, the only type of astrocyte (defined by the expression of the glial fibrillary acidic protein, GFAP) present was unable to accumulate γ-[^{3}H]aminobutyric acid (GABA), did not express surface antigens recognized by two monoclonal antibodies, A2B5 and LB1, and showed minimal proliferation. In these cultures, nonneuronal A2B5$^+$, LB1$^+$ stellate cells exhibiting "neuron-like" [^{3}H]GABA uptake formed cell colonies of increasing size and were GFAP$^-$. After about one week of culturing, the A2B5$^+$, LB1$^+$, GABA-uptake positive cell groups became galactocerebroside (GalCer) positive. Immunocytolysis of the A2B5$^+$ cells at 3 and 4 days *in vitro* prevented the appearance of the A2B5$^+$, LB1$^+$, GABA-uptake positive cell colonies, and also of the GalCer$^+$ cell groups. If 10% (vol/vol) fetal calf serum was added to 6-day cultures, the A2B5$^+$, LB1$^+$, GABA-uptake positive cell groups expressed GFAP and not GalCer. If the serum was added to the cultures 2 days after lysing the A2B5$^+$ cells, only A2B5$^-$, LB1$^-$, GABA-uptake negative astrocytes proliferated. It is concluded that the putative fibrous astrocytes previously described in serum-containing cultures (which had a stellate shape and were A2B5$^+$, LB1$^+$, GABA-uptake positive) derive from bipotential precursors that differentiate into oligodendrocytes (GalCer$^+$) in serum-free medium or into astrocytes (GFAP$^+$) in the presence of serum, while the epithelioid A2B5$^-$, LB1$^-$, GABA-uptake negative astrocytes originate from a different precursor not yet identified.**

It is not known whether the various types of cerebellar astrocytes (1) have different functions, and little is known about the cell lineage of cerebellar glia. The availability of primary cell cultures has greatly increased the possibility of studying the functional and developmental features of glial cells under controlled experimental conditions (2). In previous studies we characterized two populations of astroglial cells [identified immunocytochemically using anti-glial fibrillary acidic protein (GFAP) antibodies] in cerebellar cell cultures grown in the presence of fetal calf serum (FCS) (3–5). One cell type, often present in small colonies, had a stellate shape, while the other had an epithelioid appearance. Both avidly accumulated the nonmetabolized analog of L-glutamate, D-[^{3}H]aspartate, but only the stellate cells accumulated γ-[^{3}H]aminobutyric acid (GABA) to a substantial degree, through a transport system that was indistinguishable from that of cerebellar GABAergic interneurons, on the basis of its sensitivity to a number of GABA transport inhibitors (4, 5).

In addition to a "neuron-like" GABA transport system, stellate astrocytes expressed other membrane properties shared by neurons (5), such as surface antigens binding the monoclonal antibody A2B5 (6), and, transiently, tetanus toxin receptors (7). Raff and coworkers (8) had shown that a population of astrocytes in rat optic nerve cultures, exhibiting a shape reminiscent of our stellate astrocytes, was A2B5$^+$ and tetanus$^+$. Subsequently, these A2B5$^+$ cells were identified as fibrous astrocytes (9). The possibility that the same correspondence holds true also for the rat cerebellum is supported by data of Wilkin and Cohen (see ref. 10) who showed that a new monoclonal antibody, LB1 (obtained by immunizing mice with whole cells from embryonic rat spinal cord cultures), labels the stellate astrocytes in cerebellar cultures and cells in the putative white matter tracts in cryostat sections of developing rat cerebella.

Our autoradiographic studies on [^{3}H]GABA and D-[^{3}H]aspartate uptake by cultured cerebellar astrocytes did not provide any evidence for a developmental regulation of this function (3, 4). In contrast, studies on cultured neurons showed a clear correlation between cell differentiation and neurotransmitter-amino acid transport (11–15). The possibility that factors present in the FCS might induce an early onset of the astroglial high-affinity transport for D-[^{3}H]aspartate and [^{3}H]GABA could not be excluded in the above mentioned investigations. To analyze this problem and possibly to identify cells that might be functionally immature forms of the A2B5$^+$, LB1$^+$, GABA-accumulating astrocytes or even their precursors (16), we cultured cerebellar cells in a serum-free, chemically defined medium (CDM) and assessed their antigenic and functional features. The data obtained revealed that A2B5$^+$, LB1$^+$, GFAP$^+$ stellate cells were not present in these cultures. However, [^{3}H]GABA was accumulated by a population of proliferating A2B5$^+$, LB1$^+$ precursor cells that differentiated into oligodendrocytes if maintained in CDM or into stellate astrocytes if FCS was added to the CDM.

MATERIALS AND METHODS

Cells dissociated from 8-day-old Wistar rat cerebella after mild trypsinization (14, 17) were resuspended in CDM [3 parts of Dulbecco's modified Eagle's medium (DMEM) and 1 part of Ham's F12, containing the N2 supplements (18) and 4.6 mg of glucose/ml], cultured (2.7×10^5 cells per cm^2) on 12-mm round coverslips that had been coated with 5 μg of poly(L-lysine)/ml, placed in 35-mm dishes, and grown in the same medium at 37°C in a humidified 95% air/5% CO_2 atmosphere (17). Culture media were obtained from GIBCO, and chemicals for culture media were from Sigma.

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Abbreviations: GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; GalCer, galactocerebroside; CDM, chemically defined medium, serum-free; FCS, fetal calf serum.

For the autoradiography experiments, the coverslips were incubated for 10 min, with D-[^{3}H]aspartic acid (2 μCi/ml; 20 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) or [^{3}H]GABA (2 μCi/ml; 35 Ci/mmol; New England Nuclear), processed for autoradiography, and counterstained as described (4, 14). Exposure time was 12–16 days at 4°C. Aminooxyacetic acid was not added to the incubation medium since the pattern of [^{3}H]GABA accumulation was not changed by the presence of the inhibitor (4).

For immunofluorescence, the following procedures were adopted: (*i*) For GFAP staining (7, 19), cells were fixed in 4% (wt/vol) paraformaldehyde, permeabilized with ethanol/acetic acid, 95:5 (vol/vol) incubated with rabbit anti-GFAP serum and then with goat anti-rabbit rhodamine-conjugated IgG (Nordic, Lausanne, Switzerland), and mounted in phosphate-buffered saline, pH 7.2/glycerol (1:1; vol/vol). (*ii*) For surface staining with A2B5 (6), LB1 (10), or anti-galactocerebroside (GalCer) (20) monoclonal antibodies, cells were incubated with the antibody (A2B5 or anti-GalCer ascites fluid were diluted 1:100 in DMEM and LB1 hybridoma supernatant was used without dilution) and then with goat anti-mouse fluorescein-conjugated IgG (Miles-Yeda, Rehovot, Israel). Cells were fixed in 4% (wt/vol) paraformaldehyde. (*iii*) Double staining with rabbit anti-GFAP serum and one of the monoclonal antibodies binding to surface antigens was according to Schnitzer and Schachner (21).

For the immunocytolysis experiments with A2B5 antibodies and complement (22) cells were treated at 3 and 4 days *in vitro* with A2B5 ascites fluid (0.5 ml per dish, diluted 1:100 in CDM) for 45 min at 37°C, washed with CDM, incubated for 30 min in agarose-absorbed (23) rabbit complement (final dilution, 1:10) and washed again. In controls, CDM was used instead of A2B5 in the first step. After the last wash, the original (conditioned) culture medium was put back into the culture dishes.

RESULTS

When cerebellar cells from 8-day-old rats were cultured in CDM, granule neurons accounted for over 90% of the cells in the culture (17). The GFAP$^+$ cells were generally elongated, with long, thick processes (Fig. 1*A*), showed very low proliferation (17), were strongly labeled by D-[^{3}H]aspartate (Fig. 1*B*) and very modestly by [^{3}H]GABA (Fig. 1*C*). [^{3}H]GABA was instead avidly accumulated by cell colonies formed by a progressively increasing number of stellate cells (Fig. 1*C*) that took up also D-[^{3}H]aspartate (Fig. 1*B*). These cells were GFAP$^-$, as shown by combined autoradiography and GFAP immunofluorescence (data not shown). As in the case of GABAergic neurons, the uptake of [^{3}H]GABA into the stellate cells was insensitive to β-alanine and was abolished by *cis*-aminocyclohexane carboxylic acid (27). The monoclonal antibodies A2B5 and LB1 labeled colonies of GFAP$^-$ stellate cells similar to the cell groups accumulating [^{3}H]GABA (Fig. 2 *A*, *D*, *F*, and *G*). The number of labeled cells in these colonies increased with time in culture (Fig. 2 and Table 1) and incorporation of [^{3}H]thymidine into their nuclei (data not shown) confirmed that they proliferated. This observation and the fact that the A2B5$^+$ cells were not decorated by anti-glutamate decarboxylase (24) or by anti-microtubule-associated protein 2 (25) antibodies excluded the possibility that they were neurons, in particular GABAergic neurons, which, in addition, show a different morphology (27). A2B5 labeled also neurons, but not intensely, while LB1 did not, at the culture stages examined.

To further identify the A2B5$^+$, LB1$^+$, GABA-uptake positive cells, the cultures were stained with a monoclonal antibody reacting with GalCer (20), considered as a specific marker for oligodendrocytes (16). Up to about 6 days *in vitro* the GalCer$^+$ cells had a scattered distribution (Fig. 2*C* and Table 1). In older cultures, anti-GalCer antibodies labeled not only scattered cells, but also cell groups morphologically very similar to the groups of A2B5$^+$ or LB1$^+$ cells (Fig. 2*H* and Table 1).

To determine if the A2B5$^+$, LB1$^+$, GABA-uptake positive stellate cell colonies seen until 6 days *in vitro* are precursors of the GalCer$^+$ cell groups, cultures in CDM were treated with A2B5 and complement at 3 and 4 days *in vitro*, to lyse the A2B5$^+$ cells (22), and then examined at 6 or 10–12 days *in vitro* (Table 1). In three experiments practically no A2B5$^+$, LB1$^+$ cells nor GABA-uptake positive cell colonies (data not shown) were detectable, while the GFAP$^+$ cells were similar in number to those present in control cultures, treated with complement alone. The immunocytolysis of the A2B5$^+$ cells caused also the disappearance of the GalCer$^+$ cell groups seen to develop after 6 days *in vitro*, while the number of scattered GalCer$^+$ cells was not significantly affected.

In rat optic nerve cultures, A2B5$^+$, GFAP$^-$ precursors have been shown to express an oligodendroglial phenotype (GalCer) in CDM, and an astroglial phenotype (GFAP) in the presence of serum (16). When 10% (vol/vol) FCS was added to cerebellar cells cultured in CDM for 6 days, many A2B5$^+$, LB1$^+$ cells became GFAP$^+$ (see Fig. 3 *A* and *B*) whereas GalCer$^+$ cell groups did not develop. The A2B5$^+$, LB1$^+$, GFAP$^+$ stellate cells present in these cultures accumulated both [^{3}H]GABA and D-[^{3}H]aspartate, while the A2B5$^-$, GFAP$^+$ cells expressed an epithelioid shape and took up only D-[^{3}H]aspartate (Fig. 3).

Finally, when 10% (vol/vol) FCS was added at 6 days *in vitro* to cultures treated with A2B5 and complement at 3 and 4 days *in vitro*, only epithelioid A2B5$^-$, LB1$^-$ astrocytes lacking GABA transport developed (Fig. 4 *A* and *B*). In contrast, in controls treated with complement alone, large

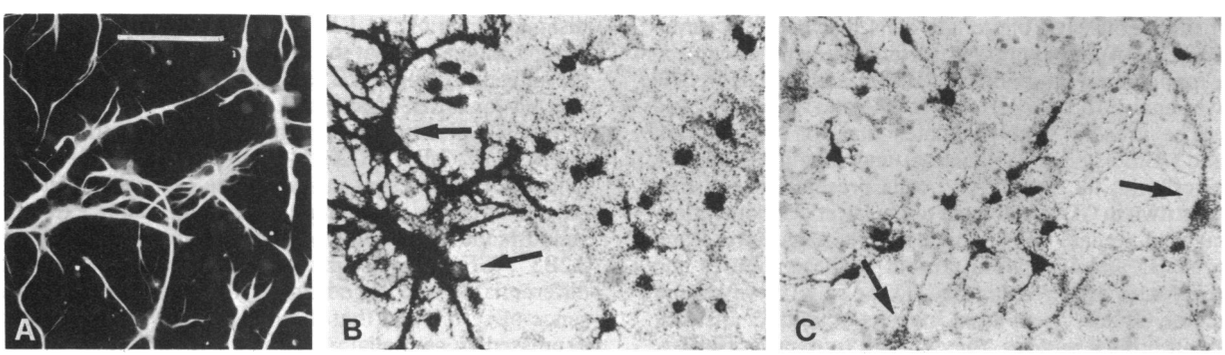

FIG. 1. GFAP labeling and autoradiographic accumulation of D-[^{3}H]aspartate and [^{3}H]GABA into cerebellar cells cultured for 12 days in CDM. (*A*) Cells labeled with anti-GFAP antibodies, rhodamine optics. (*B* and *C*) Autoradiographic uptake patterns of D-[^{3}H]aspartate, *B*, and [^{3}H]GABA, *C*, into astrocytes (arrows) and groups of small GFAP$^-$ stellate cells. Cells were incubated for 10 min with 2 μCi of ^{3}H-labeled amino acid/ml. (Bars: *A* and *B*, 82 μm; *C*, 95 μm.)

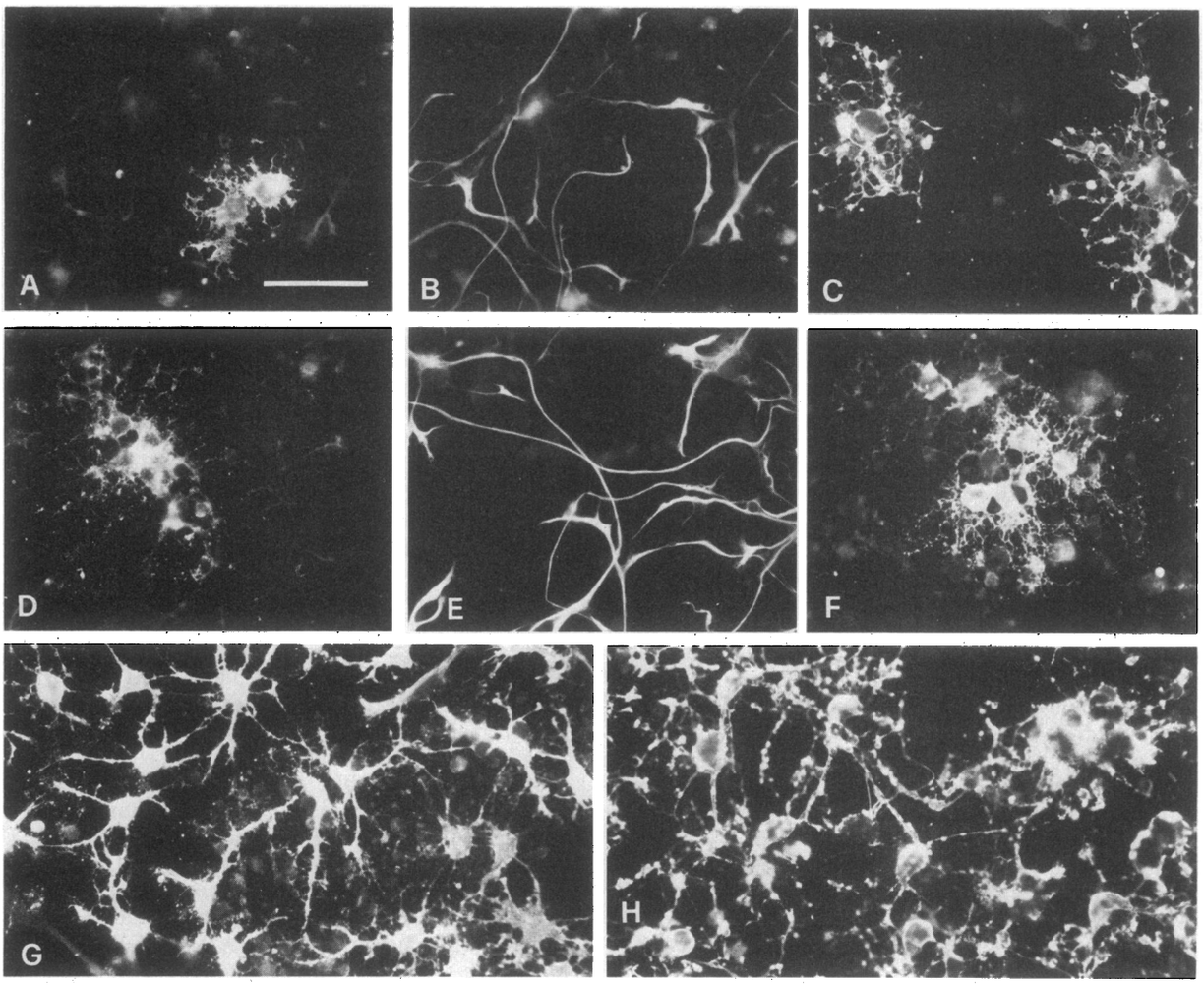

FIG. 2. Immunofluorescence labeling of cerebellar cells cultured in CDM. (*A* and *B*) Cultures at 3 days *in vitro*, double labeling with A2B5 (*A*, fluorescein optics) and anti-GFAP (*B*, rhodamine optics) antibodies, same microscopic field. (*C*) Cultures at 6 days *in vitro*, labeled with anti-GalCer antibodies (fluorescein optics). (*D* and *E*) Cultures at 6 days *in vitro*, double labeling with A2B5 (*D*, fluorescein optics) and anti-GFAP (*E*, rhodamine optics) antibodies, same microscopic field. (*F*) Cultures at 6 days *in vitro*, labeled with LB1 antibodies (fluorescein optics). (*G*) A2B5 labeling of cultures at 12 days *in vitro* (fluorescein optics). (*H*) Cultures at 12 days *in vitro* labeled with anti-GalCer antibodies (fluorescein optics). A2B5 labels stellate cell colonies of increasing size (*A*, *D*, *G*) that are $GFAP^-$ (*B*, *E*) and are labeled also by LB1 (*F*). $GalCer^+$ cells are always scattered in early cultures (*C*), but are also seen in large groups at later stages (*H*). (Bars: *A* and *B*, 52 μm; *C–H*, 82 μm.)

groups of [^{3}H]GABA accumulating cells were also seen (Fig. 4*C*). These cells bound the antibodies A2B5 and LB1 and expressed GFAP (Fig. 4 *D* and *E*).

DISCUSSION

When postnatal rat cerebellar cells were cultured in a serum-containing medium, two populations of astrocytes (defined by their GFAP content) could be distinguished (3–5). One of them consisted of stellate cells that avidly accumulated [^{3}H]GABA and expressed surface antigens recognized by the monoclonal antibodies A2B5 (5) and LB1 (10). The other was unable to transport [^{3}H]GABA and was $A2B5^-$ and $LB1^-$. The present study indicates that, on the basis of antigenic (A2B5 and LB1 labeling) and functional (GABA uptake) criteria, all the $GFAP^+$ cells present in cerebellar cultures grown in CDM belonged to the latter cell population. Nonneuronal stellate cells showing [^{3}H]GABA uptake and binding A2B5 and LB1 were present in these cultures and formed colonies of increasing size. These cells, however, did not become $GFAP^+$ even after 3 weeks of culture. Instead, after about 6 days *in vitro*, they started to express GalCer, thus acquiring an oligodendrocyte phenotype (7, 16). That GalCer was indeed expressed by the same nonneuronal cells that bound A2B5 was demonstrated by lysing the $A2B5^+$ cells with A2B5 and complement before the appearance of $GalCer^+$ cell groups. After this treatment, no $GalCer^+$ cell groups were detectable at later culture stages. Moreover, preliminary experiments in which cells cultured in CDM were double labeled with A2B5 (or LB1) and anti-GalCer antibodies, showed coexistence of A2B5 (or LB1) and GalCer in a substantial number of cells during the second week of culturing.

The immunocytolysis of the $A2B5^+$ cells led also to the disappearance of the $LB1^+$ and of the GABA-$uptake^+$ cell groups, indicating that the same nonneuronal cells expressed the antigens binding A2B5 and LB1 and the membrane carrier utilized for GABA transport.

The data discussed so far are compatible with the idea that the $A2B5^+$, $LB1^+$, GABA-uptake positive, $GFAP^-$ nonneuronal cells present in serum-free cerebellar cultures are oligodendrocyte precursors. This conclusion extends and supports the observation of Raff and coworkers (16) that the $A2B5^+$, $GFAP^-$ cells present in rat optic nerve cultures differentiated into oligodendrocytes when grown in the absence of serum and that the optic nerve $A2B5^+$ precursors expressed an astroglial phenotype (GFAP) when cultured in the presence of serum. It thus seemed conceivable that the $A2B5^+$, $LB1^+$, GABA-uptake positive, $GFAP^+$ stellate cells described in our cerebellar cultures grown with serum (3–5) could derive from the same precursors that differentiate into

Table 1. Immunofluorescent cell counts at various culture stages and after immunocytolysis with A2B5 and complement

	Coverslips, no.	Scattered cells per cm²	Cell colonies per cm²	Cells per colony
3 DIV				
A2B5	3	14 ± 3	16 ± 1	2.4 ± 0.1
LB1	6	32 ± 5	17 ± 3	2.6 ± 0.2
GalCer	4	102 ± 12	5.7 ± 2.1	2.1 ± 0.1
6 DIV				
A2B5	6	1.3 ± 0.1	16 ± 2	6.8 ± 0.6
LB1	4	1.7 ± 0.1	18 ± 2	7.0 ± 0.3
GalCer	4	84 ± 6	3.0 ± 0.9	2.2 ± 0.1
10–12 DIV				
A2B5	4	0	20 ± 2	27 ± 4
LB1	6	1.0 ± 0.3	14 ± 2	29 ± 2
GalCer	9	54 ± 10	15 ± 1	16 ± 4
11 DIV (A2B5+C)				
A2B5	5	0.3 ± 0.3	0.7 ± 0.4	22 ± 10
LB1	6	0	0.2 ± 0.1	28 ± 22
GalCer	4	82 ± 15	1.8 ± 1.1	2.3 ± 0.4
11 DIV (C)				
A2B5	3	1.5 ± 1.2	19 ± 1	19 ± 4
LB1	5	1.5 ± 0.8	19 ± 2	24 ± 5
GalCer	6	91 ± 25	15 ± 2	9.1 ± 1.1

Cells were counted on immunofluorescence-labeled coverslips, using a ×40 or a ×16 objective and a ×10 eye-piece on a Zeiss photomicroscope. Scattered cells and cell colonies were scored by counting 30–50 microscopic fields per coverslip in 3–9 coverslips from 2–5 experiments. The number of cells per colony (2 or more closely associated cells) was obtained by counting all the positive cells in 10 random cell colonies on each coverslip. In the immunocytolysis experiments [11 DIV (A2B5+C) and 11 DIV (C)] cultures were treated at 3 and 4 DIV with A2B5 antibodies and complement (C) or complement alone. At 3 DIV the number of A2B5⁺ isolated cells may be underestimated, due to the difficulty of differentiating them from neurons. The figures presented are averages of the cell counts obtained for the different coverslips ± SEM. DIV, days *in vitro*.

GalCer⁺ cells in CDM. This hypothesis is supported by the observation that when 10% (vol/vol) FCS was added to cerebellar cells cultured in CDM for 6 days, the A2B5⁺, LB1⁺ cell colonies remained GalCer⁻ but became GFAP⁺ and retained the ability to take up [³H]GABA. Moreover, adding the FCS to cultures treated with A2B5 and complement led to the proliferation of a population of GFAP⁺ cells that was A2B5⁻, LB1⁻, and GABA-uptake negative.

Although the A2B5⁺, LB1⁺ stellate cells started to express GalCer after a week of culturing in CDM, other cells were GalCer⁺ at earlier stages. These cells, however, were A2B5⁻, never appeared in groups (and, therefore, did not

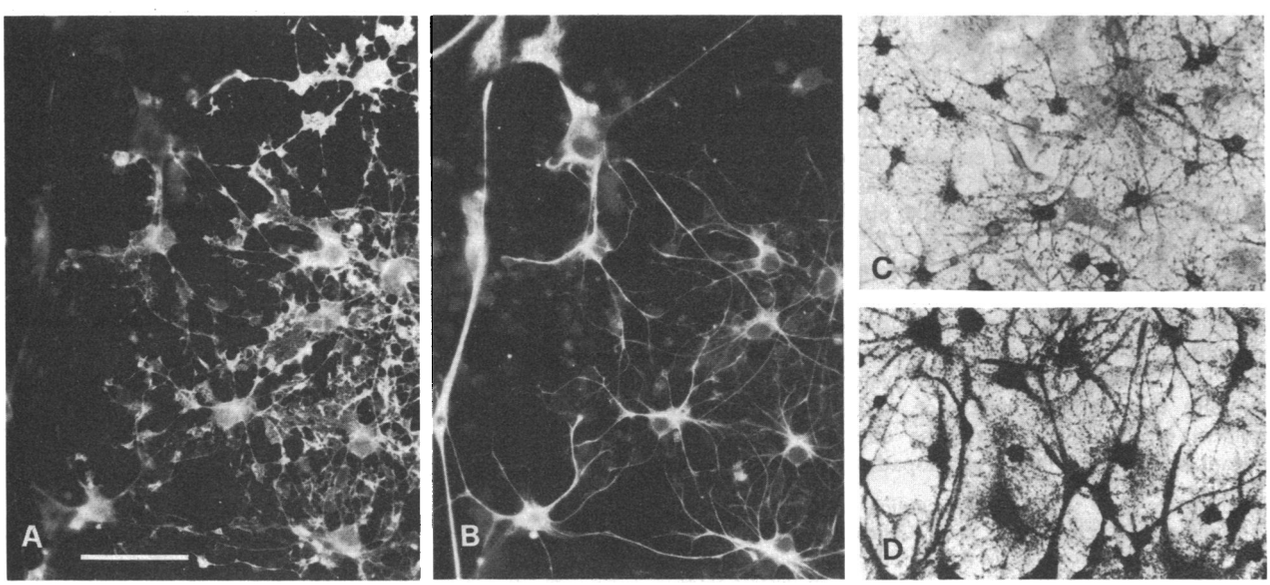

FIG. 3. Immunofluorescence labeling and autoradiographic accumulation of [³H]GABA and D-[³H]aspartate in cerebellar cells at 11 days *in vitro*, after addition of 10% (vol/vol) FCS to the CDM at 6 days *in vitro*. (*A* and *B*) Double labeling with LB1 (*A*, fluorescein optics) and anti-GFAP (*B*, rhodamine optics) antibodies, same microscopic field. Many stellate cells are stained by both antibodies. In two other experiments in which cells were labeled with A2B5 and anti-GFAP antibodies (3 coverslips), we counted randomly 500 A2B5⁺ cells. Of these, 58% were GFAP⁺. (*C* and *D*) Autoradiographic uptake of [³H]GABA, *C*, and D-[³H]aspartate, *D*, after 10 min incubation with 2 μCi of ³H-labeled amino acid/ml. Epithelioid astrocytes are not labeled by [³H]GABA. (Bars: *A* and *B*, 52 μm; *C* and *D*, 95 μm.)

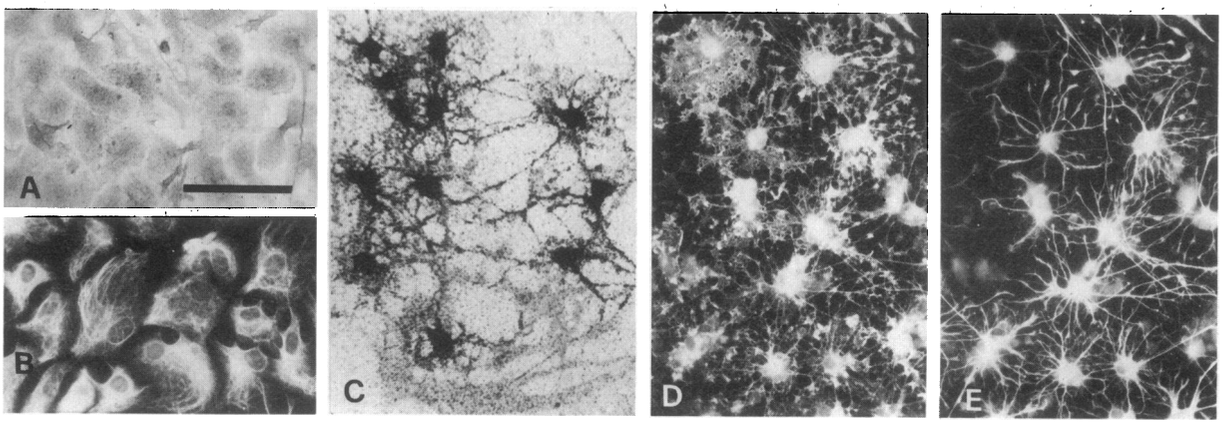

FIG. 4. Lack of [^{3}H]GABA uptake by cerebellar cells cultured in CDM, treated with A2B5 and complement, and then shifted to 10% (vol/vol) FCS. (*A* and *B*) Cultures treated at 3 and 4 days *in vitro* with A2B5 and complement. FCS (10%, vol/vol) was added at 6 days *in vitro* to the CDM, and cells were used for [^{3}H]GABA autoradiography, *A*, or for GFAP labeling, *B*, at 11 days *in vitro* (details as in Fig. 1). No stellate cells labeled by [^{3}H]GABA are present. (*C*) [^{3}H]GABA is taken up by the stellate cells growing in control cultures, treated with complement at 3 and 4 days *in vitro* and shifted to 10% (vol/vol) FCS at 6 days *in vitro*. (*D* and *E*) Double labeling with A2B5 (*D*, fluorescein optics) and anti-GFAP (*E*, rhodamine optics) antibodies of control cultures (see *C*) at 11 days *in vitro*, same microscopic field. Many stellate cells bind both antibodies. In four coverslips from two control experiments we counted randomly 350 A2B5$^+$ cells. Of these, 77% were GFAP$^+$. (Bars: *A*, 148 μm; *B*, *D*, and *E*, 82 μm; *C*, 95 μm.)

seem to proliferate), and were not affected by lysing the A2B5$^+$ cells. It is not yet known whether the scattered GalCer$^+$ cells derive from an independent A2B5$^-$ precursor or from the A2B5$^+$, oligodendrocyte/astrocyte precursor that has lost the antigen binding A2B5 as a consequence of its being postmitotic.

In conclusion, of the two types of cerebellar astrocytes characterized in serum-containing cultures (3–5), the cells expressing a stellate morphology and a number of neuron-like features (type of GABA transport, binding of A2B5 antibodies and, transiently, of tetanus toxin), which were tentatively identified as fibrous astrocytes (5, 10), appear to derive from a bipotential precursor with similar antigenic and functional properties except for the lack of GFAP. Similarly to the precursor described in the optic nerve (16), this cell can differentiate into an oligodendrocyte when cultured in the absence of serum.

It is noteworthy that the ability of transporting [^{3}H]GABA and D-[^{3}H]aspartate is already present in a nondifferentiated progenitor cell and is conserved independently of the differentiated cell type (oligodendrocyte or astrocyte) that originated from it. Our results are consistent with the observation that [^{3}H]GABA is accumulated by cultured oligodendrocytes (26).

The differentiation of the A2B5$^-$, LB1$^-$ astrocytes capable of D-[^{3}H]aspartate, but not of [^{3}H]GABA, uptake did not appear to be greatly influenced by the absence of FCS. In fact, although these cells exhibited a morphology different from that seen in the presence of FCS, they rapidly became GFAP$^+$.

In future studies it will be important to assess if bipotential oligodendrocyte/(putative fibrous) astrocyte precursors are present also in the mature nervous system, and what the factors are that induce the choice of the differentiation pattern *in vivo*. Answers to these questions may have important implications in the physiopathology of glial cells.

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