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O2A progenitor cells transplanted into the neonatal rat brain develop into oligodendrocytes but not astrocytes

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ABSTRACT **The differentiation of the bipotential O2A progenitor cell into an oligodendrocyte or a type 2 astrocyte has been well documented in cell cultures of various regions of the central nervous system. The appropriate tools to prove its existence *in vivo* have been lacking. We report on an *in vitro–in vivo* approach that combines stable labeling of an enriched population of cultured O2A progenitors by the fluorescent dye fast blue, followed by their transplantation into neonatal rat brains, which allowed us to study the influence of the brain microenvironment on their lineage decision. The grafted cells survived well and 21 days after grafting nearly all were positive for the oligodendroglial marker galactocerebroside. Surprisingly, the fast blue-positive grafted cells did not stain for the astroglial marker glial fibrillary acidic protein. These results indicate that the O2A progenitor's plasticity is restricted by the *in vivo* environment, resulting in the developmental exclusion of the type 2 astrocyte initially described *in vitro*.**

Over the last 15 years, considerable information about glial cell development and functions has been generated through studies using various glial cell culture models. A biopotential glial (O2A) progenitor, the oligodendrocyte-type 2 astrocyte progenitor (O2A cell), which can be induced to differentiate into an oligodendrocyte or a subset of astrocytes termed the type 2 astrocyte, has been initially characterized in neonatal rat optic nerve cultures by Raff and his collaborators (1, 2). Similar findings have been reported in cultures from other regions of the central nervous system (CNS) (refs. 3 and 4; for review, see ref. 5). The O2A cell is a small motile proliferative cell that bears processes and is identified by the monoclonal antibody A2B5. The O2A progenitor does not express galactocerebroside (GC) and glial fibrillary acidic protein (GFAP), respectively, markers for oligodendrocytes and astrocytes. When cultured in serum-free medium, O2A cells spontaneously develop into $GC^+/A2B5^-$ oligodendrocytes, suggesting this is the constitutive developmental pathway for these cells. In contrast, exposure of O2A progenitors to fetal calf serum induces differentiation into $A2B5^+/GFAP^+$ type 2 astrocytes. Immunoreactivity for A2B5 is absent from type 1 astrocytes, which differ in many properties from type 2 astrocytes, and originates from a separate lineage (5). Both types contain $GFAP^+$ intermediate filaments.

Recently, the existence of type 2 astrocytes *in vivo* has been questioned (6, 7). Raff and coworkers (5) have estimated the *in vivo* appearance of type 1 astrocytes, oligodendrocytes, and type 2 astrocytes in the rat optic nerve to be at postnatal day 0 (P0), P4, and P15, respectively. However, a light and electron microscopic immunocytochemical analysis of [^{3}H]thymidine pulse-labeled developing rats has revealed only two periods of astrocyte and oligodendrocyte generations, the earliest being that of $GFAP^+$ cells near P0 followed by a wave of oligodendrocytes at P4–P8 (6, 7). A major obstacle to resolve this controversy has been the lack of appropriate markers to identify type 2 astrocytes in the brain (5). The antibody A2B5 is not a reliable marker in tissue sections because it stains unrelated cell types and intracellular structures (for review, see refs. 7 and 8).

In this study, we propose an approach to compare the fate of O2A progenitors in the *in vivo* vs. *in vitro* environment. An enriched population of O2A progenitors in culture was labeled with the fluorescent dye fast blue (FB) and transplanted into neonatal rat brains. The phenotypic differentiation of O2A cells was studied in tissue sections by single and double immunolabeling and examined by fluorescence microscopy to visualize grafted cells and their progeny. We have reported (9) that FB-labeled cultured glial cells transplanted into normal and myelin-deficient (md) Wistar rats remain identifiable in immunostained tissue sections up to 6 months after grafting (9). We now report that transplanted O2A progenitors *in vivo* develop into oligodendrocytes but not astrocytes.

MATERIALS AND METHODS

Animals. Normal Wistar and md Wistar rat pups were raised in our breeding colonies. Rats were housed in a restricted-access temperature-controlled vivarium on a 12-h light/12-h dark cycle, with free access to food and water. Fifty percent of pups born to previously identified female carriers of the md trait are genetically normal ($+/y$) and 50% carry the md trait (md/y). Males of both genotypes were used at P5 as recipients for cell transplants.

Cell Culture. Primary cultures were prepared from newborn rat brains as described (10). These cultures were maintained in Waymouth medium supplemented with 10% (vol/vol) fetal calf serum. After 9 days in culture, O2A cells growing on top of the astrocyte monolayer (type 1 astrocytes) were removed by a modification of the method of McCarthy and de Vellis (11). Flasks were shaken on an orbital shaker for 10 min at 400 rpm. The medium was decanted and the cells were pelleted by centrifugation. The pellet was resuspended in culture medium, sequentially filtered through 25- and 15-μm nylon mesh, plated in four-well plates at 3×10^5 cells per well, and incubated for 4 hr. Then the medium was replaced by a serum-free chemically defined medium (10) that results in the elimination of the remaining type 1 astrocytes and macrophages. This medium consists of Dulbecco's modified Eagle's medium supplemented with penicillin (50 international units/ml), glutamine (50 μg/ml), insulin (5 μg/ml; Sigma), and bovine serum albumin (0.8 mg/ml; Sigma). Twenty hours later the medium was replaced with fresh culture medium. Three days later the cells were labeled with

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Abbreviations: FB, fast blue; GC, galactocerebroside; GFAP, glial fibrillary acidic protein; P, postnatal day; md, myelin-deficient; CNS, central nervous system.

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Table 1. Antigenic phenotype of cultured cells at the time of transplantation

Total cells, no.	O2A progenitors ($A2B5^+/GFAP^-$), no.	Type 2 astrocytes ($A2B5^+/GFAP^+$), no.	Type 1 astrocytes ($A2B5^-/GFAP^+$), no.	Unstained cells, no.
870 ± 12	710 ± 14	110 ± 3	10	60 ± 5

Total number of cells was obtained by counting cells in three areas per coverslip by using phase-contrast microscopy. Numbers represent the average ± SD of three double immunostaining experiments.

the fluorescent dye FB (Sigma) as described (9). Briefly, a sterile aqueous solution of FB dye was prepared at 10 mg/ml. The solution was kept in the dark at 4°C and diluted 1:1000 in Waymouth medium. Cell cultures were incubated in this medium for 5 hr. Then the cultures were washed with fresh culture medium and kept overnight at 37°C. The culture medium containing a few floating cells was discarded and the adhering cells were harvested by gentle scraping, pelleted by centrifugation, and resuspended in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution.

Transplantation Procedures. Five-day-old rat pups were weighed and anesthetized with Avertin. The Avertin stock solution was prepared by dissolving 25 g of 2,2,2-tribromoethanol in 15.5 ml of tetraamyl alcohol and was stored at 4°C. Before use the stock solution was diluted 1:39 in physiological saline and was injected intraperitoneally at a dose of 0.1 ml/10 g (body weight). In preparation for the injection, the skull was exposed by a short incision of the skin. A 1-mm^2 hole was bored into the skull, ≈1.5 mm lateral right and 0.75 mm caudal to the bregma. The syringe was inserted 3.5 mm deep and 10 μl of the cell suspension (2.2×10^5 cells) was injected over 5 min. The needle was left in place 5 min to allow diffusion of the cell suspension and was withdrawn stepwise over a 5-min period. When the operated pups regained consciousness, they were put back with the mother. Animals were observed daily. The developmental milestones of animals receiving cell transplants and noninjected animals were the same. The md animals started to show typical tremors on schedule at day 14 that were followed by seizures. Twenty-one days after transplantation, animals were anesthetized with Avertin at 0.2 ml/10 g (body weight). Rats were intracardiacally perfused with physiological saline at room temperature for 5–10 min at a flow rate of ≈20 ml/min. Then a fixative solution of 4% (wt/vol) paraformaldehyde in 0.1% sodium phosphate buffer (pH 7.2) was perfused at the same flow rate for 10 min. The brain was excised, sectioned midsagitally, and immersed in the same fixative. After paraffin embedding, tissue was cut into 6-μm sections. Paraffin sections were mounted on polylysine-coated slides and left overnight at 37°C. Sections were cut 30 μm thick with a Vibratome.

Immunocytochemistry. Cell cultures were prepared either on coverslips or on four-well plates for immunostaining with cell-type-specific antibodies. The cell cultures were fixed with 3.7% (vol/vol) formaldehyde on the same day that transplants were performed. The primary antibodies A2B5,

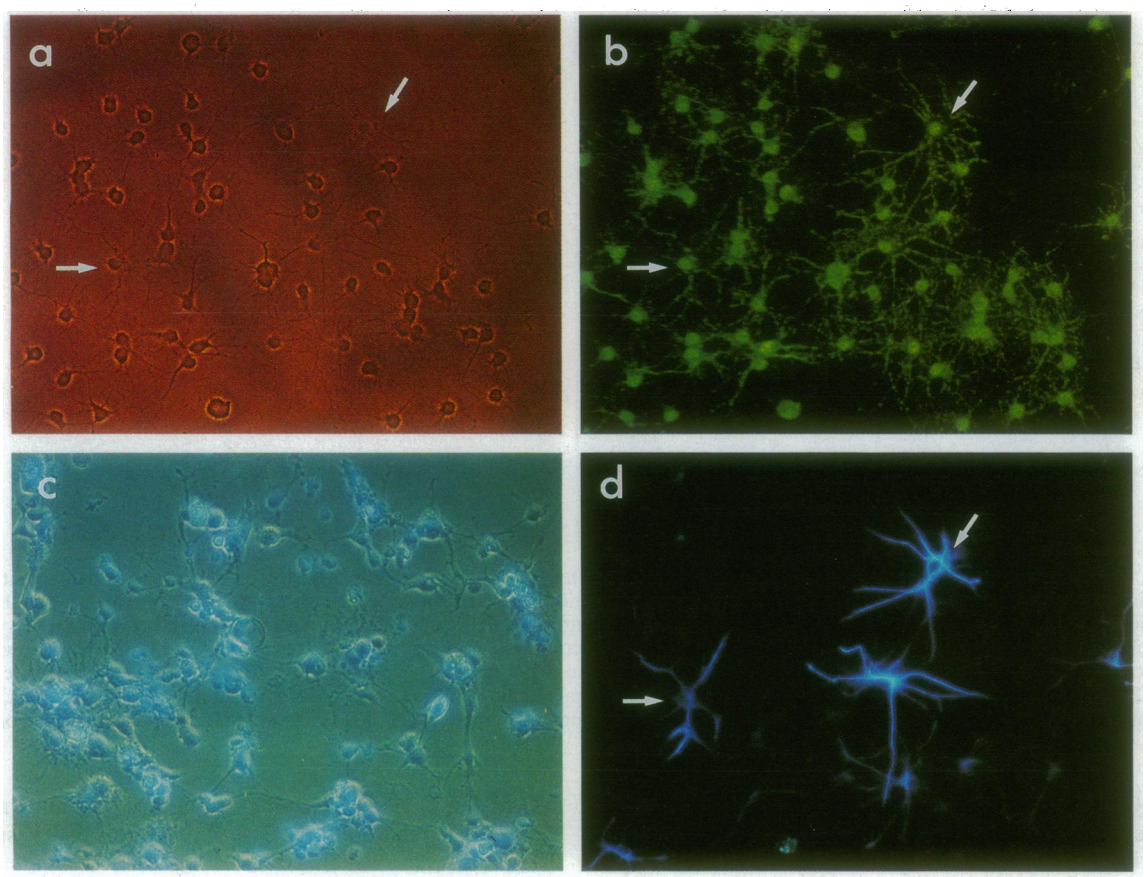

FIG. 1. Properties of purified O2A cells. (*a*) Phase-contrast morphology of O2A cells. (*b*) Immunofluorescent visualization of $A2B5^+$ cells in the same field. The majority of cells were stained (see Table 1). (*d*) Double immunostaining with anti-GFAP antiserum shows that few cells are $GFAP^+/A2B5^+$. These cells display the typical morphology of type 2 astrocytes: small round cell body with thin and long cell process. (*c*) FB^+ cells 24 hr after labeling of sister cultures with the dye. The photograph was taken at the time of transplantation. Most cells were either bipolar or multipolar and were labeled with the dye. (×120.)

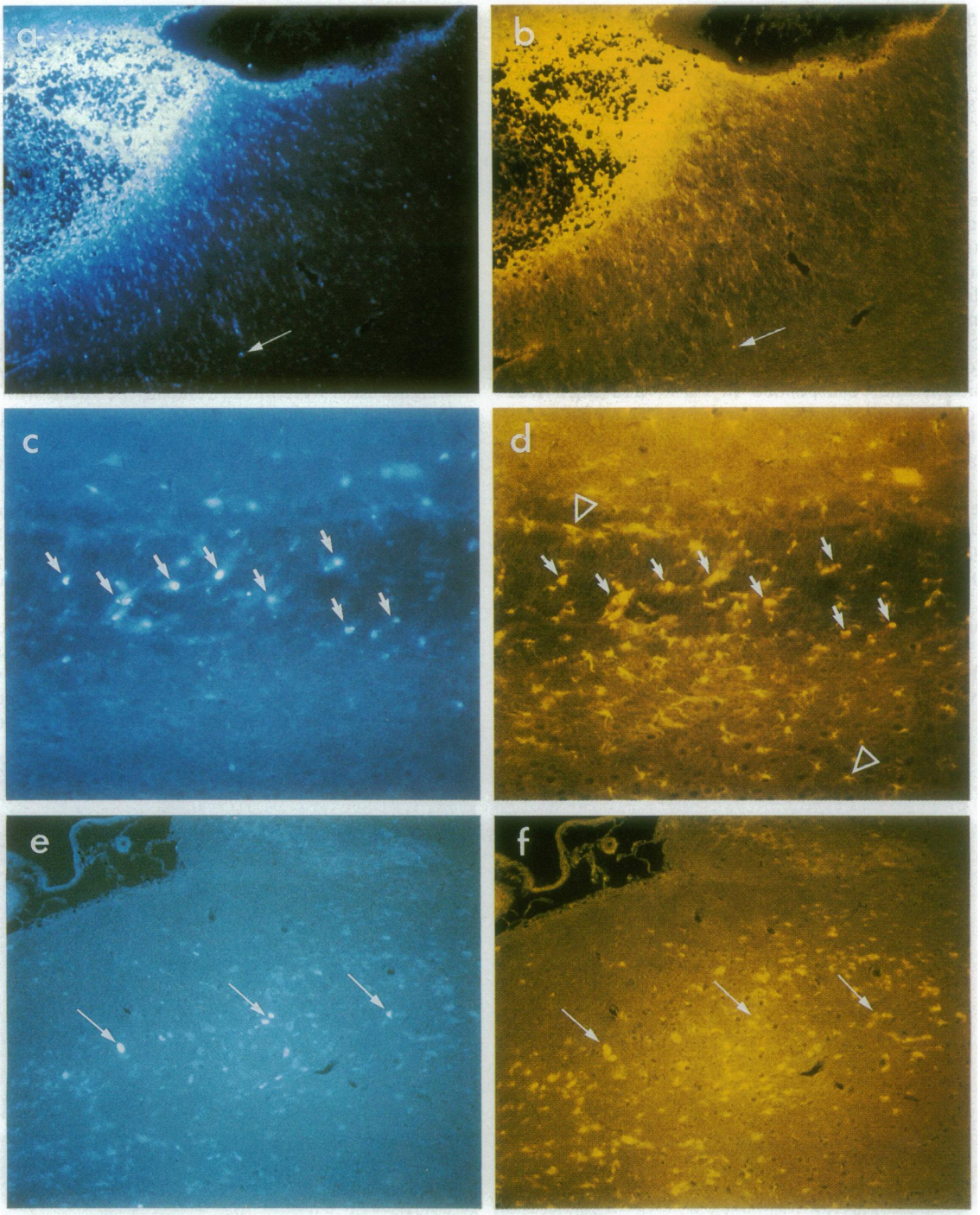

FIG. 2. Paraffin sagittal section from unaffected rat brain 21 days after transplantation. This sagittal view shows the transplant site indicated by Indian ink and the immediate surrounding area. (*a*) FB^+ cell migration in all directions can be observed in peripheral areas to the injection site. (*b*) View of the same area stained with anti-GC antibody. The pattern of FB^+ cells overlaps with GC^+ cells. (*c*) High-power view of the needle track. Arrows indicate some of the FB^+ cells. (*d*) The same section was double immunostained for GC and GFAP and viewed in triple fluorescence exposure. Cells in orange are GC^+ cells that are also FB^+ (arrows). Green-yellow (fluorescein isothiocyanate) cells are $GFAP^+/FB^-$ (arrowheads), indicating that they are host cells. A typical reactive gliosis can be observed along the needle track. (*e*) A view of the cerebral cortex. Arrows indicate FB^+ cells. (*f*) The same area was immunostained for GC. FB^+ cells were GC^+ indicating migration of grafted cells away from the transplant site. (*a* and *b*, ×60; *c–f*, ×120.)

GC, and GFAP were used at dilutions of 1:5000, 1:50, and 1:100, respectively. Microglia were identified with N2, a rabbit anti-rat vault polyclonal antibody that was a generous gift of Leonard Rome (University of California, Los Angeles). Secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Boehringer Mannheim), 7-amino-4-methylcoumarin-3-acetic acid-conjugated goat anti-mouse IgG and IgM (Jackson Immunochem, West Grove, PA), and rhodamine-conjugated goat anti-rabbit (Sigma) used at dilutions of 1:350, 1:100, and 1:500, respectively. Markers were visualized by using double or triple immunofluorescence techniques.

We have previously reported that single or double immunofluorescence of tissue sections allowed us to identify host FB^- and grafted FB^+ cells (9). All immunostained cell cultures and tissue sections were analyzed with a Nikon FX

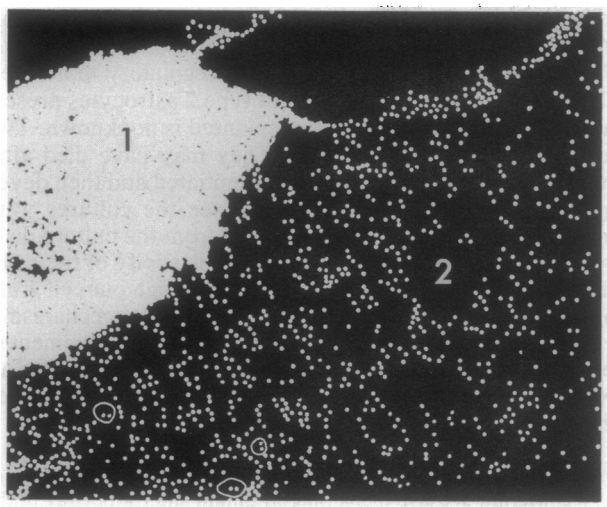

FIG. 3. Camera lucida image of FB$^+$ cells in representative sagittal section (Fig. 2*a*). In this partial view of a sagittal section, zone 1 is the graft site and zone 2 is the area around the injection site. The total number of cells in zones 1 and 2 was 157,200 ± 15,700 and 40,413 ± 3640, respectively. (×120.)

microscope equipped with the appropriate filters and barriers. Kodak Ektachrome 400 ASA daylight slide film was used. Color prints for preparing publication plates were obtained from such color slides.

Cell Counts. The total number of FB$^+$ cells present 21 days after transplantation was estimated from 200 serial 6-μm paraffin sections. One in five sagittal sections was counted and the cell count was adjusted by taking into account the average oligodendrocyte cell body diameter, reported to be 7 μm (12). The marginal zones were clearly evident by the decreased number of FB$^+$ cells compared to the 200 serial sagittal sections, which displayed a similar density of FB$^+$ cells. These marginal zones comprising 18 sections were excluded from the cell count.

RESULTS

An enriched population of O2A progenitor cells was obtained from primary mixed glial cultures by combining the methods of McCarthy and de Vellis (11) and Espinosa de los Monteros *et al.* (10). O2A progenitors were identified as A2B5$^+$/GFAP$^-$ cells. They constituted 80% of the cells (Table 1) grafted into brains of unaffected and 5-day-old md rat pups. GFAP immunostaining was almost solely identified with type 2 astrocytes (Fig. 1 and Table 1). Type 1 astrocytes and microglia were removed by our cell culture procedures. FB dye was taken up and retained by all cultured cells (Fig. 1*c*). We have shown (9) that FB is not cytotoxic and does not alter the differentiation of glial cells *in vitro* and *in vivo* after transplantation. *In vivo*-grafted cells expressed GC, glycerol phosphate dehydrogenase, and myelin basic protein (9). The dye does not pass into other cells and can be visualized by fluorescence microscopy for >6 months. Although cell proliferation results in dilution of the dye, FB$^+$ cells show intense labeling after six cell divisions in culture (9). Thus FB dye appears to be one of the best cell markers at the light microscopic level for tracking single cells and studying their differentiation and fate in the *in vivo* microenvironment.

The transplantation site was easily identified 21 day after transplantation by the Indian ink that was coinjected with the cell suspension (Fig. 2 *a* and *b*). The majority of FB$^+$ cells were confined to the injection site and its vicinity that are called zones 1 and 2, respectively, in Fig. 3. FB$^+$ cells also migrated to distant sites in both normal (Fig. 2*e*) and md host brains (data not shown). Single FB$^+$ cells or clusters of four to six cells were observed at distant sites in the cerebral cortex (Fig. 2 *e* and *f*), myelinated tracts, and cerebellum. In all instances, nearly all FB$^+$ cells were GC$^+$, as shown in Fig. 2. This surprising finding suggested that O2A progenitors differentiate into GC$^+$ oligodendrocytes but not GFAP$^+$ type 2 astrocytes in the *in vivo* environment. To confirm this possibility, GC/GFAP double immunostaining was systematically performed on all sections analyzed. Examples of double immunostaining are shown for needle-track areas in unaffected (Fig. 2*d*) and in md (Fig. 4*b*) rats. GFAP immunostaining was never observed in FB$^+$ cells. The needle-track area was selected to illustrate the contrast between host GFAP$^+$/FB$^-$ astrocytes and FB$^+$/GFAP$^-$ grafted cells. Single GFAP immunostaining provided direct evidence that FB$^+$ grafted cells do not express GFAP (Fig. 4*a*). These results show that grafted O2A cells survived, migrated, differentiated, and integrated into host tissue. At 21 days after grafting, 201,679 ± 22,184 FB$^+$ cells were counted, a number almost equal to the total number of cells injected. The majority of FB$^+$ cells was found at the injection site and the surrounding zone (Figs. 2 and 3). A small percentage of cells, 4056 ± 486 cells, was found at various distant sites principally in the cerebral cortex (Fig. 2*e*) and white matter tracts (data not shown). To investigate the possibility that macrophages/microglia may become FB$^+$ by phagocytosis of FB-labeled cells, sections were immunostained with the N2 anti-vault

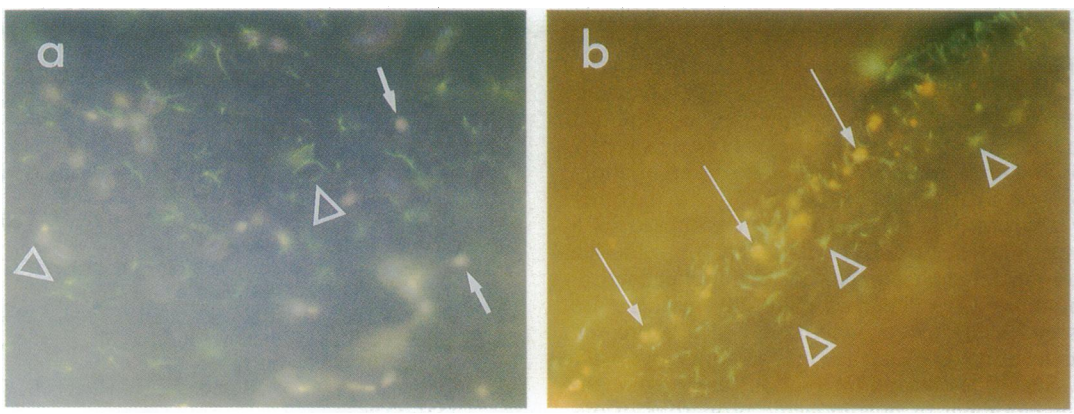

FIG. 4. Vibratome sections (30 μm) 21 days after transplantation of O2A cells into rat brains. (*a*) Section from unaffected rat brain. FB$^+$ cells are GFAP$^-$ (arrows). GFAP$^+$ cells are FB$^-$ (arrowheads). (*b*) View of a section from a md rat brain. Triple exposure micrograph of double immunofluorescence was taken at the level of the needle track. Orange (rhodamine plus FB) fluorescence shows that the cells are GC$^+$/FB$^+$. Green (fluorescein isothiocyanate) shows that these cells are solely GFAP$^+$ and do not contain FB. The host GFAP$^+$ cells delineate the needle track. (×140.)

antibody, a specific marker for microglia and macrophages (13). No FB^+ microphages were detected (data not shown).

DISCUSSION

Transplantation of neural tissue fragments and dissociated cells into the CNS has been largely used as a potential approach to stimulate regeneration and recovery of function after injury associated with neurological disorders and trauma. Most of the studies involving glial cells have been performed in dysmyelinating mouse and rat mutants (14). In these animals oligodendrocytes generally survive but fail to myelinate axonal tracts. Interestingly, the transplantation of developing brain tissue or a suspension of mixed populations of CNS cells results in extensive myelination, indicating that normal oligodendrocytes can differentiate in these md mutants and that the defect is largely intrinsic to host oligodendrocytes (14, 15). Similar results have been obtained using the md rat mutant (16, 17), which is of interest to us because it originated from the same rat strain that was used to prepare O2A progenitor cultures. This model allows integration of grafted cells into the host CNS without apparent rejection for at least 6 months (9). This mutant is characterized by a point mutation in exon 3 of the *PLP* gene (18) and displays an arrest in oligodendrocyte differentiation (19, 20). The advantage of using myelin mutants in transplantation studies is that the myelination observed must come from the grafted cells. Hence this approach obviated the need to label cells. An increasing number of fluorescent dyes have recently become available to label cells. The use of the dye FB has been proven in our experience to be a stable and effective cell marker to visualize and study the phenotype of grafted cells in both normal and md mutant animals (9).

This approach combined with the purification of an enriched population of O2A progenitor cells allowed us to ask whether these cells are bipotential in the *in vivo* microenvironment as they are under appropriate conditions *in vitro* (1, 2). The O2A progenitors were grafted at P5, a time when these cells are known to be present *in vivo* (8). Type 2 astrocytes have been estimated to arise 7–10 days later (8). Since we did not observe the generation of $GFAP^+$ cells from grafted O2A progenitors in normal and md rats, we surmise that the event is rare, transient, or occurs under specific circumstances. The cell culture conditions in which type 2 astrocytes are obtained may be considered an injury model since cells in the normal brain are not exposed to serum and, therefore, may not be exposed to the GFAP-inducing factor(s) present in serum. Other possibilities include the lack of appropriate paracrine and cell–cell contacts *in vitro* that *in vivo* might restrict the expression of GFAP in O2A cells. In high-density cell culture of mixed primary glia, only a small proportion of O2A lineage cells express GFAP mRNA (21). This effect may be mediated by interactions with astrocytes (22). Dutly and Schwab (23) have reported that conditioned medium from neonatal cultures of astrocytes restricts type 2 differentiation and promotes oligodendrocyte differentiation, whereas embryonic astrocytes lack this activity. On the other hand, ciliary neurotrophic factor has been proposed as a signal for type 2 astrocyte differentiation (24). Autocrine and/or contact-mediated phenomena may favor oligodendrocyte differentiation. Oligodendrocyte mRNA markers are highly expressed in cell clusters, whereas GFAP is expressed in dispersed cells of the O2A lineage in high-density primary cultures (21). Specific adhesion interactions are a prerequisite for the development of myelinogenic properties of oligodendrocytes (25), but it is not known whether such factors are also involved in lineage decision. Further experiments *in vitro* are needed to identify potential regulatory molecules. The fate of the small population of type 2 astrocytes present in the population of cells we transplanted is not known. Two possibilities can be considered. They may have died after transplantation or may have dedifferentiated and then developed into GC^+ oligodendrocytes. Since the cultures were immature and the cells were detached from the culture dish, we feel these conditions increased the plasticity of the cells, hence their ability to dedifferentiate. Although our cell grafting model cannot be used to identify molecular mechanisms, it should be very useful to investigate the developmental, regional, or pathological conditions that may regulate GFAP expression (i.e., type 2 astrocyte formation) in the O2A cell lineage in the *in vivo* microenvironment.

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1. Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R. & Noble, M. (1983) *J. Neurosci.* **3**, 1289–1300.
2. Raff, M. C., Miller, R. H. & Noble, M. (1983) *Nature (London)* **303**, 390–396.
3. Levi, G., Gallo, V. & Ciotti, M. T. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1504–1508.
4. Ingraham, C. A. & McCarthy, K. D. (1989) *J. Neurosci.* **9**, 63–69.
5. Miller, R. H., French-Constant, C. & Raff, M. C. (1989) *Annu. Rev. Neurosci.* **12**, 517–534.
6. Skoff, R. (1990) *Dev. Biol.* **139**, 149–168.
7. Skoff, R. P. & Knapp, P. E. (1991) *Glia* **4**, 165–174.
8. Noble, M. (1991) *Glia* **4**, 157–164.
9. Espinosa de los Monteros, A., Zhang, M., Gordon, M., Aymie, M. & de Vellis, J. (1992) *Dev. Neurosci.* **14**, 98–104.
10. Espinosa de los Monteros, A., Roussel, G., & Nussbaum, J. L. (1986) *Dev. Brain Res.* **24**, 117–125.
11. McCarthy, K. D. & de Vellis, J. (1980) *J. Cell Biol.* **85**, 8790–8902.
12. Mori, S. & Leblond, C. P. (1970) *J. Comp. Neurol.* **139**, 1–30.
13. Chugani, D. C., Kedersha, N. L. & Rome, L. H. (1991) *J. Neurosci.* **11**, 256–268.
14. Blakemore, W. F. & Franklin, R. J. M. (1991) *Trends Neurosci.* **8**, 323–327.
15. Lachapelle, F., Gumpel, M., Baulac, M., Jacque, C., Duc, P. & Baumann, F. (1983/1984) *Dev. Neurosci.* **6**, 325–334.
16. Rosenbluth, J., Hasegawa, M., Shirasaki, N., Rosen, C. L. & Liu, Z. (1990) *J. Neurocytol.* **19**, 718–730.
17. Duncan, L. D., Hammang, J. P., Jackson, K. F., Wood, P. M., Punge, R. P. & Langford, L. (1988) *J. Neurocytol.* **17**, 351–360.
18. Boison, D. & Stoffel, W. (1989) *EMBO J.* **8**, 3295–3302.
19. Kumar, S., Macklin, W. B., Gordon, M. N., Espinosa de los Monteros, A., Cole, R., Scully, S. A. & de Vellis, J. (1990) *Dev. Neurosci.* **12**, 316–325.
20. Kumar, S., Gordon, M. N., Espinosa de los Monteros, A. & de Vellis, J. (1988) *J. Neurosci. Res.* **21**, 268–274.
21. Holmes, E., Hermanson, G., Cole, R. & de Vellis, J. (1988) *J. Neurosci. Res.* **19**, 389–396.
22. Aloisi, F., Agresti, C., D'Urso, D. & Levi, G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6167–6171.
23. Dutly, F. & Schwab, M. E. (1991) *Glia* **4**, 559–571.
24. Lilien, L. E., Sendtner, M., Rohrer, M., Hughes, S. M. & Raff, M. C. (1989) *Neuron* **1**, 485–494.
25. Cardwell, M. C. & Rome, L. H. (1988) *J. Cell Biol.* **107**, 1551–1559.