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De novo generation of neuronal cells from the adult mouse brain

(neuronal precursors/fibroblast growth factor/neurofilament/differentiation)

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ABSTRACT **Cells of neuronal morphology, expressing the 150- and 200-kDa neurofilament proteins, were generated *in vitro* from populations of neural cells dissociated from adult (>60-day-old) mouse brain. Most of these neurons arose from dividing precursors, as demonstrated by the incorporation of [^{3}H]thymidine during the culture period and autoradiography. Neuronal production was optimal under the conditions in which precursors were initially stimulated with basic fibroblast growth factor and then exposed to medium conditioned by an astrocytic cell line, Ast-1, in serum-free medium. Few, if any, neurons arose in control cultures or in cultures kept in serum and fibroblast growth factor. These results suggest that neuronal precursors exist in the adult mammalian brain, but they require discrete epigenetic signals for their proliferation and differentiation.**

The neuronal and glial elements of the central nervous system are generated from precursor cells in the neuroepithelium during early development (1). The majority of neurons are formed, with exceptions, such as granule neurons in the hippocampus (2), by birth in the mouse. Although glial cells in the adult mouse are known to be replaced either from precursor cells (3) or by proliferation of existing glia (4), there is no evidence for the presence of neuronal precursors within the mouse central nervous system. However, olfactory epithelium derived from embryonic placode has been demonstrated to retain the ability to generate olfactory neurons in adult mammals, including mice (5). Further, some adult birds retain the ability to generate new neurons, often in response to hormonal stimuli, from precursors in their ventricular zone; the neurons then migrate and populate central nervous system nuclei controlling birdsong learning (6, 7). As we had recently identified factors that regulate precursor proliferation and differentiation in developing murine neuroepithelium (8), we decided to re-examine the possibility that the adult mammalian brain contained neuronal precursors, recognizing that discrete epigenetic factors might be necessary to stimulate their proliferation and subsequent differentiation.

We report the *de novo* generation *in vitro* of neurofilament-containing cells of neuronal morphology from a precursor population obtained from the adult central nervous system. This response is dependent on basic fibroblast growth factor (bFGF), which appears to stimulate precursor proliferation as indicated by the incorporation of [^{3}H]thymidine into most neurons. Induction of maximal neuronal differentiation, however, was only achieved after removal of serum and bFGF and exposure of the precursors to a medium conditioned by the astrocyte-precursor cell line Ast-1.

MATERIALS AND METHODS

Isolation of Adult Brain Cells. Adult CBA/CaH WEHI mice, with an average age of 65 days, were killed with CO_2, and their brains were removed and placed into Hepes-buffered Eagle's medium (HEM). The brains were then trimmed by two coronal cuts: one was just behind the olfactory bulbs, and the second was anterior to the cerebellum. The resulting brain segment, containing the cerebral cortex, hippocampus, diencephalon, striatum, and septum, was used. The segments were chopped finely by using a sterile scalpel blade and then placed into 20 ml of HEM with 0.1% (wt/vol) trypsin (Boehringer Mannheim) and 0.001% (wt/vol) DNase (Calbiochem) for 30 min at 37°C for enzymatic dissociation. The enzymatic reaction was stopped by adding 2 ml of fetal bovine serum (FBS), and the cells were washed twice in HEM/1% FBS/0.001% (wt/vol) DNase. Cells were then collected by centrifugation and resuspended in 5 ml of Dulbecco's modified Eagle's medium (DMEM)/10% FBS and gently passed through hypodermic needles ranging in size from 18 to 24 gauge. A further 5 ml of DMEM/10% (vol/vol) FBS was added before plating the cells. The total viable cell count from this isolation procedure was an average of $\approx 10^6$ cells per brain.

Cell Culture. The cells were plated at $\approx 6 \times 10^4$ cells per well in DMEM/10% FBS onto glass coverslips (13 mm; round; Deckglaser, F.R.G.), in a final volume of 1 ml. When used, growth factors were added immediately after plating. The factors used were as follows: purified human epidermal growth factor (EGF) (from Bob Whitehead, Ludwig Institute) at a final concentration of 20 ng/ml and recombinant bFGF (Boehringer Mannheim) at a final concentration of 20 ng/ml. The cells were cultured without change of medium for 7 days to allow maximal cell adhesion to the coverslip. The wells were then washed and refed with DMEM/10% FBS, and fresh growth factors were added. Cells were then cultured for 24 hr before [^{3}H]thymidine (0.03 μCi/ml; 1 Ci = 37 GBq) was added to each well and cultured for 5 more days.

After 5 days in culture with [^{3}H]thymidine, cells from some cultures were removed from the coverslips by using a trypsin/Versene solution (0.1% trypsin; Commonwealth Serum Laboratories, Melbourne, Australia), washed, and either plated directly onto irradiated Ast-1 monolayers (see below) in 8-well slides (Lab-Tek/Nunc) in serum-free DMEM or plated onto polyornithine (100 μg/ml)-coated glass coverslips at a cell density of 10,000 cells per coverslip or left on their original coverslip. Some coverslips were then inverted over the Ast-1 monolayers but separated from them by a 1.5-mm glass rod in serum-free DMEM. Thus the cells received continuously conditioned medium from the monolayer without being in direct cell contact with the monolayer. As controls, some coverslips remained in DMEM/10% FBS plus or minus the factors, and some coverslips were placed in serum-free DMEM without any growth factors or conditioned medium. All cultures were incubated for another 6 days, then fixed, and stained as below.

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Abbreviations: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

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Ast-1 Cell Line. Confluent monolayers of an astrocyte-like cell line (Ast-1) were formed by plating the cells in DMEM/10% FBS on plastic 24-well (Falcon) plates or plastic 8-well slides (Lab-Tek/Nunc). The Ast-1 monolayers were then irradiated with 40 Gy and transferred to serum-free DMEM just before use. The Ast-1 cell line was produced in our laboratory from neural cells isolated from embryonic day-18 CBA mice by retroviral infection with the protooncogene N-*myc* and selected by neomycin resistance, by described methods (9, 10). Ast-1 cells have an astrocytic morphology and can express glial fibrillary acid protein (GFAP).

Immunohistochemistry and Autoradiography. At the end of the culture period the cells were fixed in 100% methanol for 30 min at −20°C. The coverslips or 8-well slides were then washed three times in phosphate-buffered saline/1% (vol/vol) FBS and stained with either rabbit antineurofilament antibody (anti-NF), (anti-150 kDa or anti-200 kDa; Chemicon; both used at 1:400 dilution) or rabbit anti-GFAP antibody (Dako, Glostrup, Denmark; 1:100 dilution) for 30 min. Coverslips were then washed three times in phosphate-buffered saline/1% FBS and stained with sheep anti-rabbit antibody conjugated with fluorescein isothiocyanate (Silenus, Victoria, Australia) and again washed before they were dehydrated and mounted with the cells facing upward in DePeX (Gurr, Merck, Australia)-mounting medium. The coverslips were allowed to dry overnight and were then dipped in NTB2 emulsion (Kodak) and stored for 2 weeks at 4°C. After photographic developing, the cells were mounted in phosphate-buffered saline/glycerol, 1:9, containing 2.6% 1,4-diazobicyclo-(2,2,2)-octane (DABCO; Merck) to prevent quenching. The cultures were examined by fluorescence microscopy, and the number of neurofilament or GFAP-positive cells with and without [^{3}H]thymidine labeling was quantitated.

RESULTS

Presence of Neurons and Astrocytes in Adult Central Nervous System Cultures. After plating, cultures were difficult to observe by phase microscopy for the first 7 days before medium change because of myelin and other cellular debris. However, after medium change, careful examination by phase microscopy revealed very few cells (<200) in any wells and no cells with neuronal morphology. As ≈10^4 viable cells were originally plated per well, most cells clearly either did not survive or did not attach to the substrate. Only 10 days after plating was there evidence of cell proliferation, which usually occurred in geographically discrete areas in each individual well. When cultures containing serum for the entire period were examined by fluorescence microscopy for expression of 150-kDa neurofilament, regardless of factors added, very few neurofilament-positive cells were evident (Table 1). Cultures containing bFGF, with or without EGF, had, on average, the most neurofilament-positive cells (an average of 4.5 per coverslip), whereas cultures containing EGF alone or serum alone had <1 per field (an average of 0.5 per coverslip). A similar number of neurofilament-positive cells were obtained when the cultures were stained for the 200-kDa neurofilament protein. In spite of the low number of neurons, cultures containing serum plus EGF and/or bFGF did contain many GFAP-positive cells, which was ≈10 times the number seen with serum alone (Table 1).

To determine whether the serum might have inhibited differentiation of neurons, as has been reported (11) in the peripheral nervous system, cultures were transferred to serum-free conditions after 13 days. However, the number of neurofilament-positive neurons generated was still very small; on average, only 1.5 neurons were present in cultures previously treated with bFGF (Table 1).

In an attempt to increase the number of neurofilament-positive neurons generated in these cultures, coverslips containing the adult cells were transferred after 13 days of culture to serum-free medium and inverted over a monolayer of Ast-1 cells for another 6 days. This procedure enhances the differentiation of embryonic neuroepithelial cells into neurons (T.J.K. and P.F.B., unpublished work). Under these conditions an increased number of neurofilament-expressing cells was seen in populations previously exposed to either bFGF or bFGF plus EGF (Table 1). The neurofilament-positive cells arising with Ast-1 monolayers were of various morphologies, but all had long cell processes consistent with a neuronal phenotype (Fig. 1). This potentiation was not seen when factor-primed cells were plated directly onto Ast-1 monolayers.

Are Neurons Generated from Dividing Precursors? To exclude the possibility that the neurons in these cultures were merely survivors from the original isolation procedure, the cultures were incubated with [^{3}H]thymidine before exposure to the Ast-1-conditioned medium. Fig. 1 shows that some neurofilament-expressing cells in bFGF-stimulated cultures were shown to be labeled by autoradiography, indicating that they had arisen from a dividing population. A quantitative analysis revealed that, in fact, most neurofilament-positive cells were labeled (Table 1). The morphologies of the labeled and unlabeled neurons were similar, suggesting that even the unlabeled cells may have arisen from similar precursors but

Table 1. Generation of neurons and glia from adult mouse brain

Experimental conditions		Neurofilament-positive cells*		GFAP-positive cells	
Initial 13 days	Final 6 days	Total†	[^{3}H]Thymidine labeled	Total†	[^{3}H]Thymidine labeled
EGF/bFGF	EGF/bFGF	4	2	332	324
bFGF	bFGF	5	4	326	314
EGF	EGF	0	0	232	230
No factor	No factor	1	1	40	30
EGF/bFGF	SF	2	1	23	23
bFGF	SF	0	0	18	12
No factor	SF	1	0	43	29
EGF/bFGF	Ast-1, SF	20	14	72	57
FGF	Ast-1, SF	44	26	128	109
EGF	Ast-1, SF	2	2	142	127
No factor	Ast-1, SF	0	0	89	78

SF, serum-free medium.
*Cultures were stained with α-150-kDa neurofilament antibodies.
†Cells per coverslip; because of variation with cell survival between experiments the numbers shown are from individual experiments but are representative of three separate experiments.

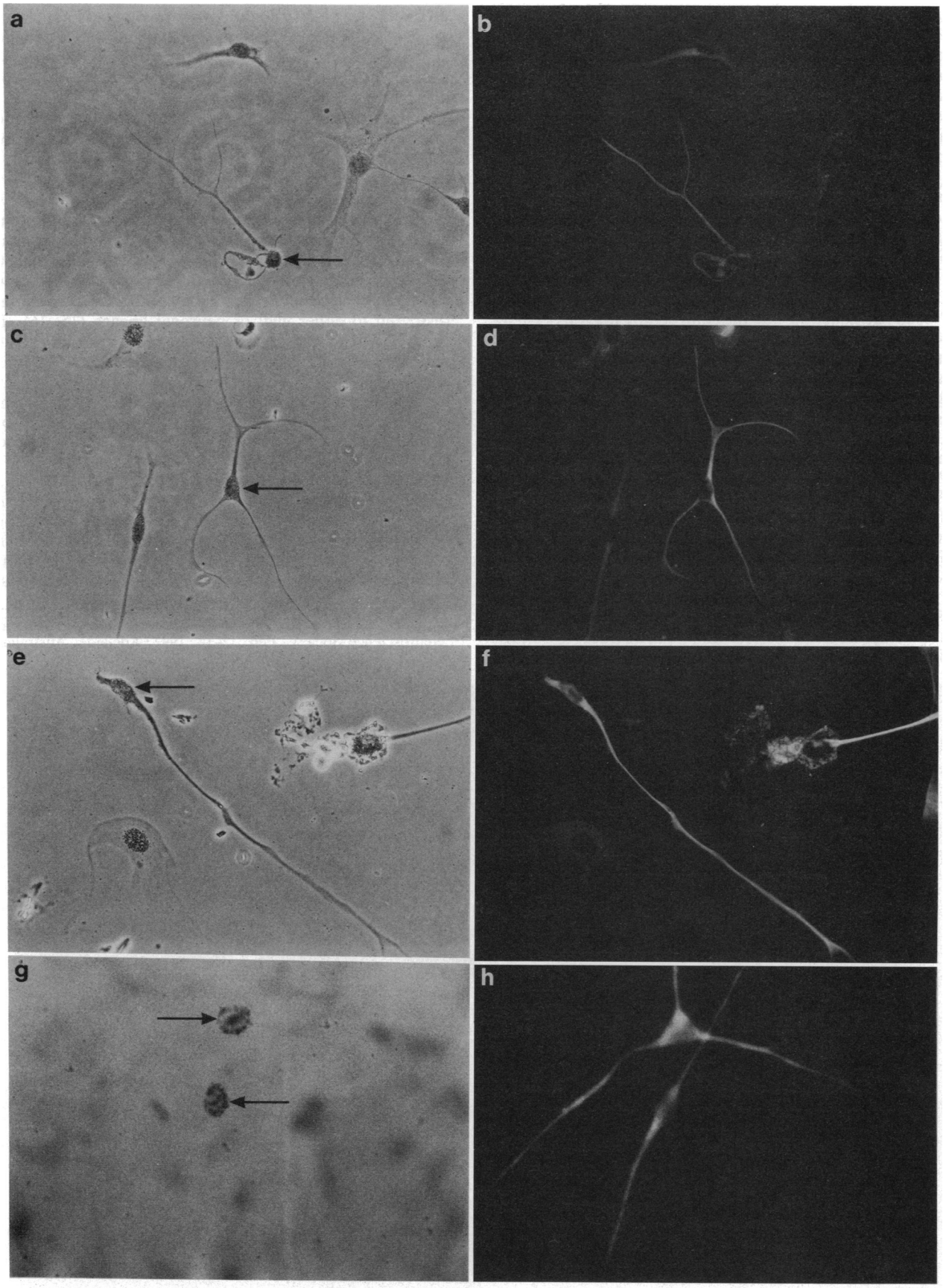

FIG. 1. Morphology of neurons generated by culturing adult brain cells with bFGF and then with medium conditioned by Ast-1 cells. Neurons stained by immunofluorescence for expression of 150-kDa neurofilament (*b*, *d*, *f*, and *h*) have various morphologies and, as shown by phase-contrast micrography, their nuclei are labeled with [^{3}H]thymidine (arrows in *a*, *c*, *e*, and *g*). The silver grains are more easily seen in *g*, where the plain of focus is at the emulsion level. (*a–f* ×280; *g* and *h* ×450.)

without cell division. Nearly all the GFAP-positive cells (86%) seen in these cultures were labeled with [^{3}H]thymidine (Table 1 and Fig. 2). Cells not staining for GFAP or neurofilament, which represented most of the cells, also were predominantly labeled.

Regional Distribution of Precursors. To further examine the distribution of these precursors within the brain, single-cell suspensions were prepared from various regions of the original brain segment. Neurofilament-positive cells arose in cultures derived from diencephalon, hippocampus, cerebral cortex, and segments containing both striatum and septum. This growth was again optimal when cultures were treated with bFGF and Ast-1-conditioned medium.

DISCUSSION

The results demonstrate that the adult central nervous system does contain a number of neural precursors, which when treated with growth factors, such as bFGF, can divide and ultimately differentiate into what appear by morphology and antigenic markers to be mature neurons. It seems unlikely that these cells represent another neural cell lineage, such as glia, as they could be identified with either the anti-150-kDa or anti-200-kDa neurofilament antibodies, did not stain with GFAP antibodies and were morphologically distinct from GFAP-positive cells.

The identity of these precursor cells is unknown, and the frequency of these cells is difficult to estimate given the low plating efficiency of this procedure. However, assuming only one precursor per coverslip—which is most unlikely, given that labeled neurons were found in all bFGF-treated cultures—it would appear that the frequency is, at least, 1 in 200 cells. *In situ*, this frequency would be considerably lower, as no dissociated neurons appear to survive the experimental procedures. The distribution of the precursor is not clear but appears to be found in all regions examined. Future studies should be able to determine whether it resides in the ventricular layer, as is the case with some birds (6, 7), or is distributed throughout the neuropil.

This study shows that the precursor cells that give rise to the majority of neurons are capable of cell division; however, the extent of their proliferation or self-renewal is still unknown. If self-renewal does occur, this would imply the existence of a more primitive type of precursor cell with multipotential differentiation capabilities. Multipotential cells are present in embryonic neuroepithelium of the central nervous system, as recently shown by retroviral tagging (12) and single-cell cloning experiments (13, †). However, with increased developmental age the precursor cells appear more restricted to either glial or neuronal lineage (15, 16). This observation suggests that either the multipotential cells disappear or become dormant and nondividing and, therefore, unable to be labeled with retrovirus. An example of the latter phenomenon comes from the hemopoietic system, where primitive multipotential stem cells have been shown to be normally nondividing and to require special stimuli to recommence cell division (17).

The precursors, whatever their differentiation potential, do require stimulation with growth factors to give rise to neurons. bFGF was far more effective in increasing neuronal-cell number compared with EGF; and this result was similar to results previously obtained with embryonic neuroepithelium, in which bFGF was the most potent stimulator of cell division

†Kilpatrick, T. J. & Bartlett, P. F., Proceedings of the Australian Neuroscience Society, Feb. 12–14, 1992, Adelaide, Australia, p. 197.

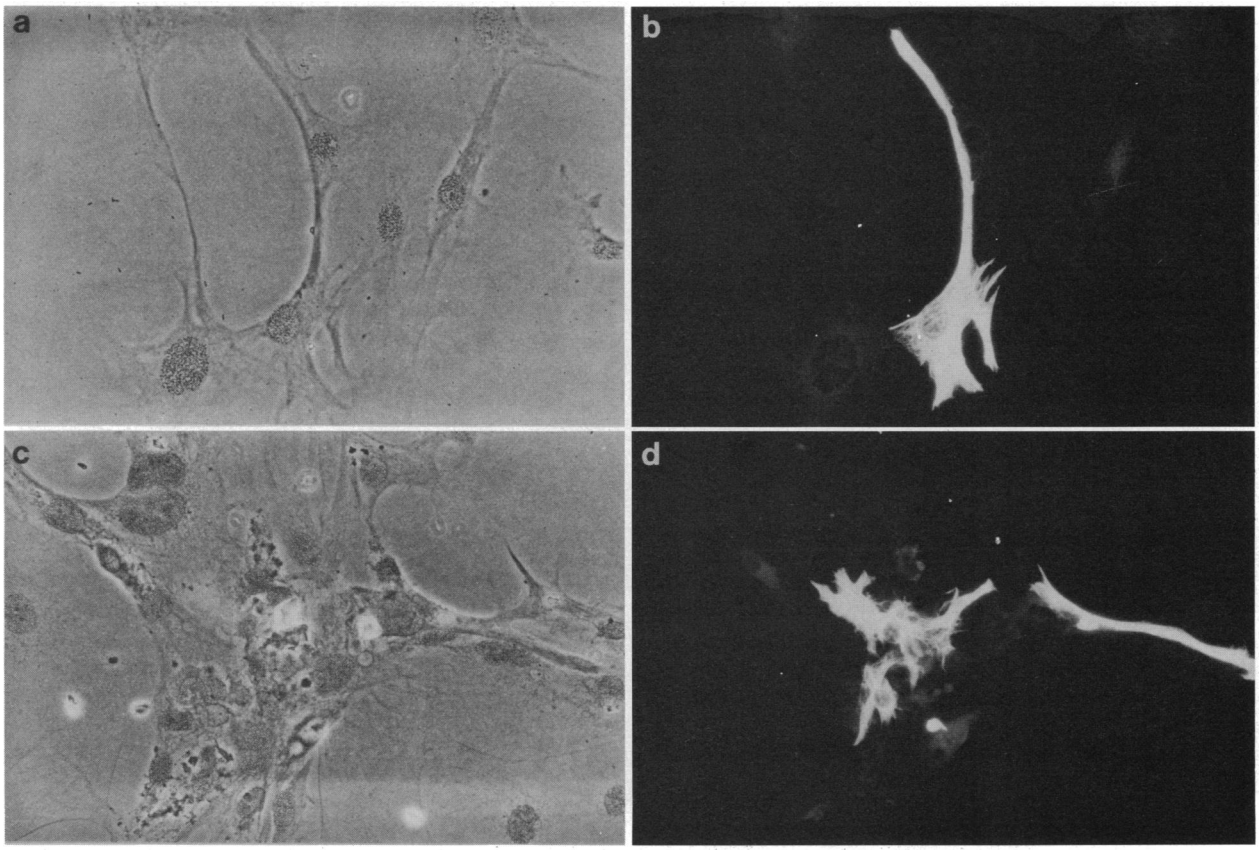

FIG. 2. Appearance of astrocytes in cultures of adult neural cells stimulated with bFGF and then with Ast-1-conditioned medium. Phase-contrast micrographs (*a* and *c*) reveal that nearly all cells are labeled with [^{3}H]thymidine; however, only a small proportion of cells are stained by immunofluorescence with anti-GFAP antibody (*b* and *d*). (×280.)

(8). Recent results have confirmed the efficacy of bFGF in stimulating neuronal precursors *in vitro* (T.J.K., unpublished data). As we have previously shown bFGF to be a mitogenic agent for neuroepithelial precursors (8) rather than a survival agent (18), its role in this system seems also to promote cell division of the precursor in adult brain. In addition, bFGF can promote neuronal differentiation in precursor cell lines (8), suggesting that bFGF may also influence commitment of the precursors toward the neuronal-cell lineage. In this system, however, another factor, provided by Ast-1, is clearly required for optimal neuronal differentiation.

Of interest was the finding that cell contact between Ast-1 and precursor cells inhibited neuron generation, suggesting that cell-surface-matrix, or adhesion, molecules are not responsible for the promotion seen with conditioned medium. This latter finding may indicate that *in situ* cell contact between the precursor and cells, such as glia, may inhibit neuronal differentiation, and this activity may explain the failure to detect neuronal turnover in the adult. Ast-1-derived neuronal-stimulatory molecules have also been shown to induce the differentiation of embryonic neural precursors (T.J.K., unpublished data), and the activities for both embryonic and adult cells could represent the same molecule.

The most exciting ramification of these findings is the possibility of stimulating the neuronal precursors *in situ* to replace diseased or damaged neuronal tissue. This activity, as indicated above, may require multiple stimuli and perhaps the neutralization of inhibitory factors, but it, nevertheless, may provide an alternative to procedures such as neural transplantation.

Note Added in Proof. Since submission of this manuscript, a paper has appeared by Reynolds and Weiss (14) that describes the generation *in vitro* of neurons from the adult mouse striatum, using ECF as the growth stimulant.

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1. Abney, E. R., Bartlett, P. F. & Raff, M. C. (1981) *Dev. Biol.* **83,** 301–310.
2. Altman, J. & Bayer, S. A. (1990) *J. Comp. Neurol.* **301,** 365–381.
3. Wolswijk, G. & Noble, M. (1989) *Development* **105,** 387–400.
4. Pruss, R. M., Bartlett, P. F., Gavrilovic, J., Lisak, R. & Rattray, S. (1981) *Dev. Brain Res.* **2,** 19–36.
5. Monte Graziadei, G. A. & Graziadei, P. P. C. (1979) *J. Neurocytol.* **8,** 197–213.
6. Alvarez-Buylla, A., Theelen, M. & Nottebohm, F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8722–8726.
7. Noordeen, E. J. & Nordeen, K. W. (1989) *Dev. Brain Res.* **49,** 27–32.
8. Murphy, M., Drago, J. & Bartlett, P. F. (1990) *J. Neurosci. Res.* **25,** 463–475.
9. Bartlett, P. F., Reid, H. H., Bailey, K. A. & Bernard, O. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3255–3259.
10. Bernard, O., Reid, H. H. & Bartlett, P. F. (1989) *J. Neurosci. Res.* **24,** 9–20.
11. Ziller, C., Dupin, E., Brazeau, P., Paulin, D. & Le Douarin, M. N. (1983) *Cell* **32,** 627–638.
12. Walsh, C. & Cepko, C. L. (1992) *Science* **255,** 434–440.
13. Temple, S. (1989) *Nature (London)* **340,** 471–473.
14. Reynolds, B. A. & Weiss, S. (1992) *Science* **255,** 1707–1710.
15. Price, J. & Thurlow, L. (1988) *Development* **104,** 473–482.
16. Williams, B. P., Read, J. & Price, J. (1991) *Neuron* **7,** 685–693.
17. Metcalf, D. & Moore, M. A. S. (1971) in *Haemopoietic Cells,* eds. Neuberger, A. & Tatum, E. L. (North Holland, Amsterdam), pp. 70–271.
18. Drago, J., Murphy, M., Caroll, S. M., Harvey, R. P. & Bartlett, P. F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2199–2203.