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Growth of purified astrocytes in a chemically defined medium

(central nervous system tissue culture/growth regulation)

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ABSTRACT **Astrocytes purified from primary cultures of neonatal rat cerebrum can now be grown in a synthetic medium supplemented with putrescine, prostaglandin $F_{2\alpha}$, insulin, fibroblast growth factor, and hydrocortisone. These five supplements have a marked synergistic effect on growth when used in combination but have little effect when used individually. Astrocytes grown in the defined medium exhibit dramatic changes in morphological characteristics in comparison to cells grown in serum-free or serum-supplemented medium. In addition, these cells express the astrocyte-specific marker glial fibrillary acidic protein and are estimated by several criteria to be greater than 95% astrocytes.**

One of the central tasks of neurobiology is to elucidate the regulatory mechanisms that underlie neural proliferation and differentiation. Studies of neuroglial physiology commonly use tissue culture techniques but have been limited by two major factors—the necessity of growing cells in the presence of serum and an inability to obtain pure populations of untransformed, primary cell cultures. Serum, a common additive to tissue culture media, contains growth factors and hormones that could potentially alter or mask the action of other compounds being examined. To eliminate the presence of these extra usually unidentified factors, a chemically defined (CD) medium was developed (1, 2).

CD media were initially developed for the growth of clonal cell lines (3) but have since been adapted for use in primary cultures as well (4). CD media developed for neural cultures have been limited primarily to the growth of clonal cell lines (5–7). In the few isolated cases where CD media have been used for primary neural cultures, there has been the problem of having more than one cell type present (8–11). An exception to this is a report by Bottenstein *et al.* describing the production of purified (95%) neuronal cultures derived from dorsal root ganglia (12). However, these cultures are not composed of a proliferating population of cells. Recently, a technique has been developed that allows for the preparation of nearly pure, separate cultures of oligodendrocytes and astrocytes (13), thus removing the second major obstacle to future research. In this communication we report that purified monolayers of rat astrocytes can proliferate in a CD medium containing hydrocortisone, putrescine, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), insulin, and fibroblast growth factor (FGF).

MATERIALS AND METHODS

Cell Culture. Purified cultures of astrocytes were prepared from primary cerebral cultures by the method of McCarthy and de Vellis (13). Primary cultures were initially plated in Falcon plastic tissue culture flasks (75 cm^2) and maintained in Ham's F-12 medium/Dulbecco–Vogt modification of Eagle's medium, 1:1 (vol/vol), with 1.2 g of $NaHCO_3$ per liter, 15 mM Hepes buffer [serum-free (SF) medium], and 10% (vol/vol) fetal calf serum. After removal of oligodendrocytes from primary cultures, the astrocytes were trypsinized with a 1% Enzar-T (40 times concentrated) trypsin concentrate and 0.1 mM EDTA in Hanks' balanced salt solution (Ca^{2+} and Mg^{2+} free) and then diluted 1:4 in medium containing 10% fetal calf serum. The cells were counted in a Royco 927TC cell tissue counter and plated at 5×10^4 cells per 35-mm Petri dish (Lux) in serum-supplemented medium. All cultures were maintained in humidified 5% CO_2/95% air at 37°C.

CD Medium. This consisted of the SF medium plus 50 nM hydrocortisone, 100 nM putrescine, $PGF_{2\alpha}$ (500 ng/ml), insulin (50 μg/ml), and FGF (100 ng/ml).

Cell Growth and Dose–Response. Approximately 1 day (18–20 hr) after plating the purified astrocytes, the serum-supplemented medium was removed, and the cells were washed twice with a SF medium. After the final wash, the experimental medium was added. This was either (*i*) SF medium, (*ii*) SF medium supplemented with 10% fetal calf serum, or (*iii*) CD medium. This was designated as day 0. Additional medium changes were always performed on day 3 and day 5. At the appropriate time, cell density was determined by trypsinization, followed by counting in a Royco cell counter. Values are reported as cell number per 35-mm Petri dish and represent the mean of triplicate or quadruplicate plates. The dose–response to individually added supplements was determined by assaying cell number at the end of a 5-day incubation.

Glial Fibrillary Acidic Protein (GFAP). Cell cultures were grown on 15-mm glass coverslips contained in microwell plates (Falcon). Cells were seeded at 1×10^4 per well (2.1 cm^2) and raised with the same protocol used for measuring the growth and dose–response curves. Five days later, astrocyte cultures were washed with phosphate-buffered saline (pH 7.4) and fixed at room temperature for 15 min in 2% (wt/vol) paraformaldehyde (0.1 M sodium cacodylate buffer/0.05% calcium chloride, pH 7.4) followed by acetone for 5 min. The cells were incubated for 30 min with anti-GFAP rabbit antiserum (1:200 dilution), washed extensively, and then stained with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (1:50 dilution; 30 min). After additional washing, the coverslips were rinsed in distilled water and mounted on glass slides with glycerol/phosphate-buffered saline, 1:9 (vol/vol). Cells were observed through a Zeiss fluorescence microscope equipped with FITC filters and mercury-vapor epi-illumination.

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Abbreviations: CD, chemically defined; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; SF, serum-free.

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Materials. Materials were obtained from the following sources: crystalline bovine insulin, $PGF_{2\alpha}$, PGE_1, sheep prolactin, fetuin, human transferrin, and putrescine dihydrochloride from Sigma; FGF and multiplication-stimulating activity from Collaborative Research (Waltham, MA); hydrocortisone acetate, L-thyroxine, progesterone, and testosterone from Calbiochem–Behring; F-12 medium (H-17), Dubecco's modified Eagle's medium (H-12), and Hanks' balanced salt solution (Mg^{2+} and Ca^{2+} free) from GIBCO; fetal calf serum from Irvine Scientific (Irvine, CA); trypsin from Reheis (Kankakee, IL); and fluorescein isothiocyanate-conjugated swine anti-rabbit IgG from Bio-Rad. The following gifts are acknowledged: anti-GFA protein rabbit antiserum supplied by L. F. Eng (Stanford University and V.A. Hospital, Palo Alto), fibronectin supplied by J. E. Bottenstein (University of California at Los Angeles), insulin supplied by M. Root (Eli Lilly), and anti-fibronectin rabbit antiserum supplied by H. R. Herschman (University of California at Los Angeles).

RESULTS

When grown in SF medium, astrocytes either grew at a slow rate or maintained a constant number before decreasing. Fig. 1 shows the fastest rate of growth observed for astrocytes grown in SF medium. After an initial lag period, some proliferation occurred, but the number of cells failed to double after 6 days in culture. In the presence of CD medium, however, the cells reached a density that was 75% of that found with serum-supplemented medium and proliferated exponentially without the lag that is found for cells switched to SF medium (Fig. 1).

Each of the individual supplements in CD medium exerted little influence on cell proliferation, with the exception of $PGF_{2\alpha}$, which elicited an 88% increase in cell number at the end of a 5-day treatment (Fig. 2). In combination the supplements acted synergistically, producing a 3-fold increase in cell number over SF medium. The absence of any one supplement resulted in cell yields that were significantly reduced with respect to those obtained in the presence of all five supplements.

The relationship between concentration of a given supplement and cell number is shown in Fig. 3. Optimal concentrations were found for each supplement with the exception of hydrocortisone. Although the dose–response curve for hydrocortisone (not shown) was highly variable from one experiment

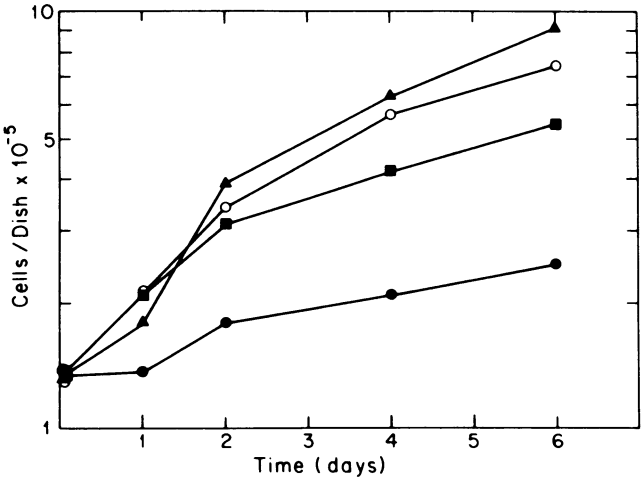

FIG. 1. Growth curve for astrocytes. Cells were grown in one of the following media: serum-free (●), serum-supplemented (▲), CD (○), or CD medium minus hydrocortisone (■). Data represent cell number per 35-mm dish; day 0, end of the serum preincubation. SEM was less than 10% for all points.

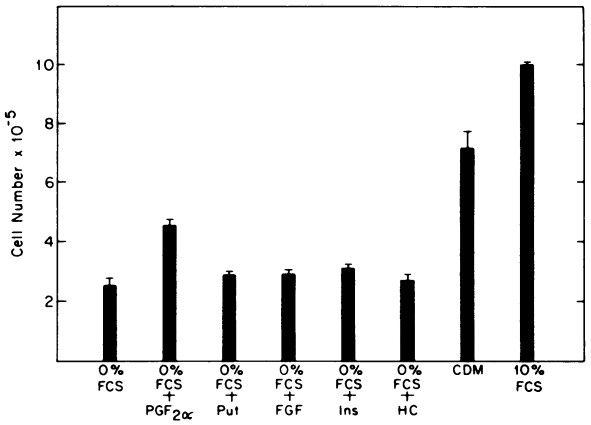

FIG. 2. Response to individual supplements. Cells were plated at 5×10^4 per 35-mm dish in serum-supplemented medium. Eighteen hours later, the cells were washed and converted to SF medium, SF medium plus one of five supplements, CD medium (CDM; all supplements), or serum-supplemented medium [10% fetal calf serum (FCS)]. The medium was changed on day 3. On day 5 cultures were trypsinized, and the cells were counted; values are expressed as cell number per 35-mm dish. Put, putrescine; Ins, insulin; HC, hydrocortisone.

to the next, we observed a consistent increase in cell number with 50 nM hydrocortisone.

The cells grown in CD medium were then identified as astrocytes by using the labeling of GFAP as an astrocyte marker (14) (Fig. 4). In 10 random fields, 95% of the cells stained positive for GFAP. Meningeal cell cultures were tested and found to be negative for GFAP (data not shown). However, meningeal cells have been shown to express fibronectin on their outer surface both *in vivo* and in culture (15, 16). These cultures were found to be positive for fibronectin as determined by immunofluorescence with anti-fibronectin rabbit antiserum (1:160 dilution; swine anti-rabbit IgG-FITC, 1:50). Less than 1% of the cells from astrocyte cultures in CD medium were positive for fibronectin (data not shown). The few cells in Fig. 4 that do not stain positive for GFAP could be meningeal contaminants or immature astrocytes that have not yet begun to synthesize GFAP.

Astrocytes grown in CD medium underwent a dramatic change in morphological characteristics in comparison to cells grown in either SF or serum-supplemented medium (Fig. 5). At the end of a 6-day incubation, cells grown in SF or serum-supplemented medium possessed few processes and appeared to be flat and polygonal shaped, whereas the majority of cells grown in CD medium had smaller cell bodies with many long, branching processes. This dense intermingling of fibers can be better observed in Fig. 4A. We maintained cultures in CD medium for more than 2 wk and found that the cells retained this differentiated appearance.

To further eliminate potentially conflicting influences of serum, we attempted to forego the 18- to 20-hr serum preincubation period. Preliminary experiments showed that when astrocytes were plated directly into SF medium, the number of cells after 24 hr in culture was only one-third of the number found in serum-supplemented cultures (Table 1). The addition of five supplements that comprise CD medium increased this number by 62%. Attachment was drastically improved by adding fibronectin (5 μg/ml). Interestingly, at the concentration tested, fibronectin increased attachment by the same order of magnitude for cells plated in SF or CD medium. In combination, fibronectin and the five supplements brought the levels of attachment of astrocytes to within 20% of those observed in the presence of 10% fetal calf serum.

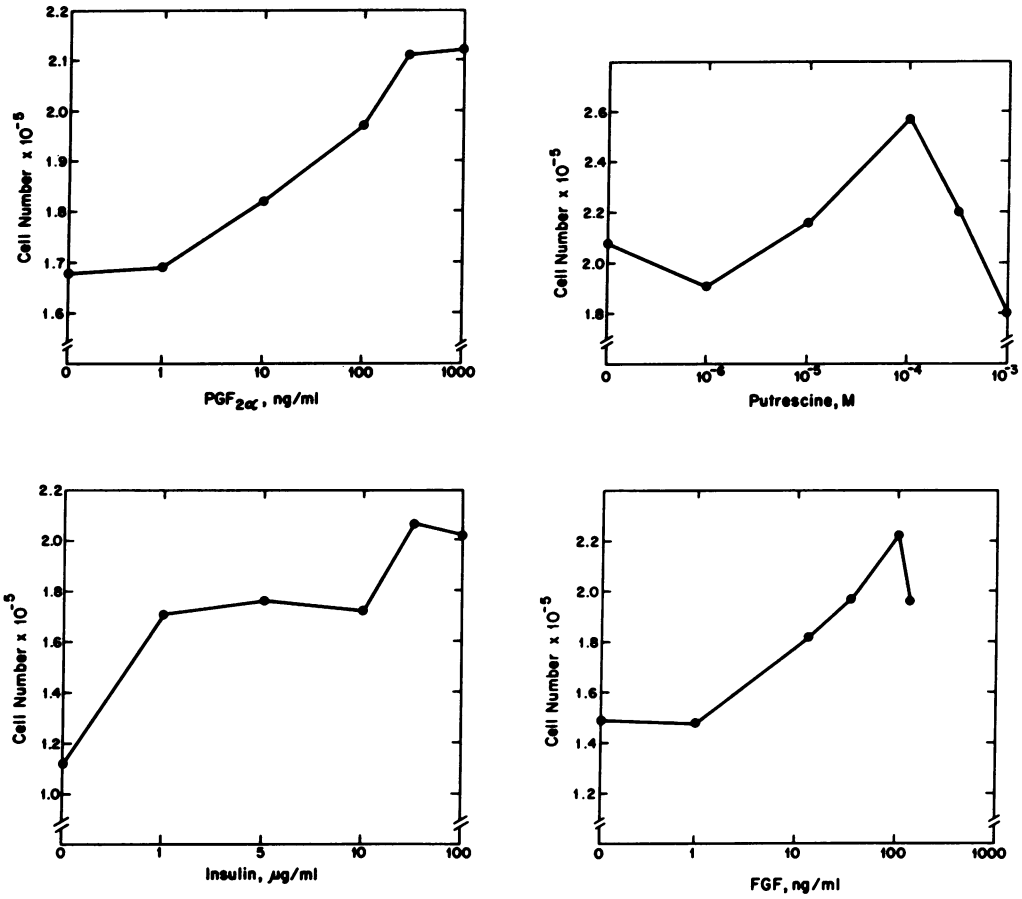

FIG. 3. Growth response as a function of supplement concentration. In each experiment, the concentration of four of five supplements was held constant while the concentration of the fifth was varied over the range indicated. SEM was less than 10% for all points.

DISCUSSION

Astrocytes purified from primary cultures of rat cerebrum can now be grown and maintained in a CD medium. Although other CD media have been reported for neural tissue, most of these support the growth of cloned cell lines derived from glial or neuronal tumors (5, 7). The N2 medium developed by Bottenstein and Sato (7) for B104 rat neuroblastoma cells has been used to support the growth of dissociated primary cultures derived from central nervous system and peripheral nervous system tissue (8–10), but these latter cultures are typically composed of more than one cell type.

The large number of available mitogens and growth-promoting agents made choosing potential factors for CD medium difficult. Our rationale was to test compounds which clearly had

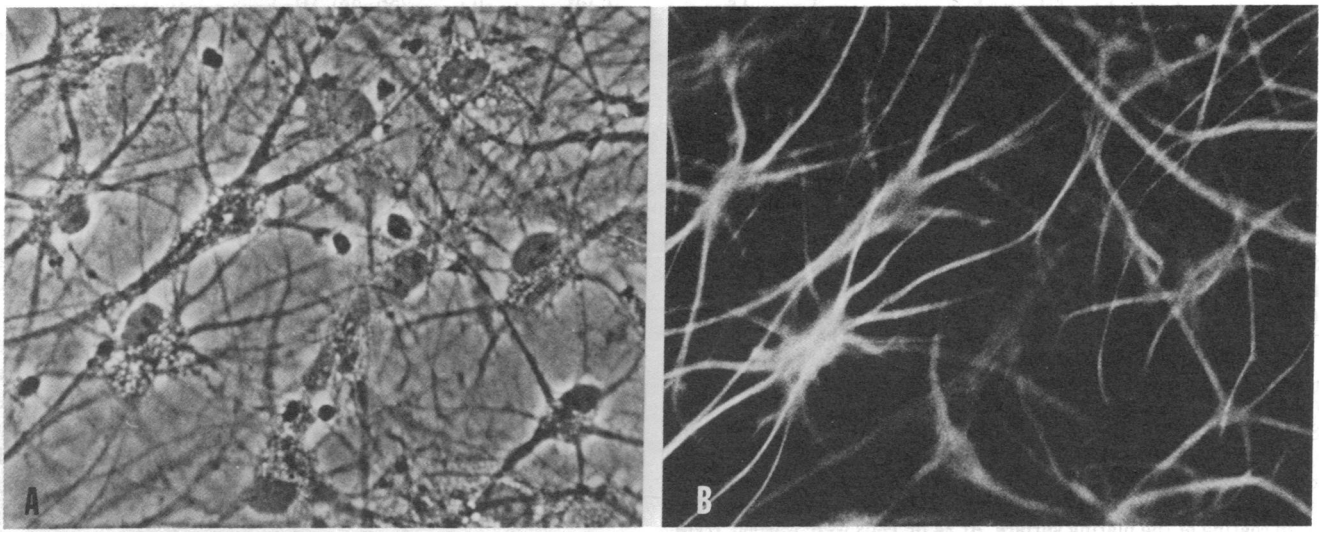

FIG. 4. GFAP immunofluorescence of astrocyte cultures grown in CD medium. The same field from a representative culture was visualized by phase-contrast microscopy (*A*) and indirect immunofluorescence for GFAP (*B*). (×500.)

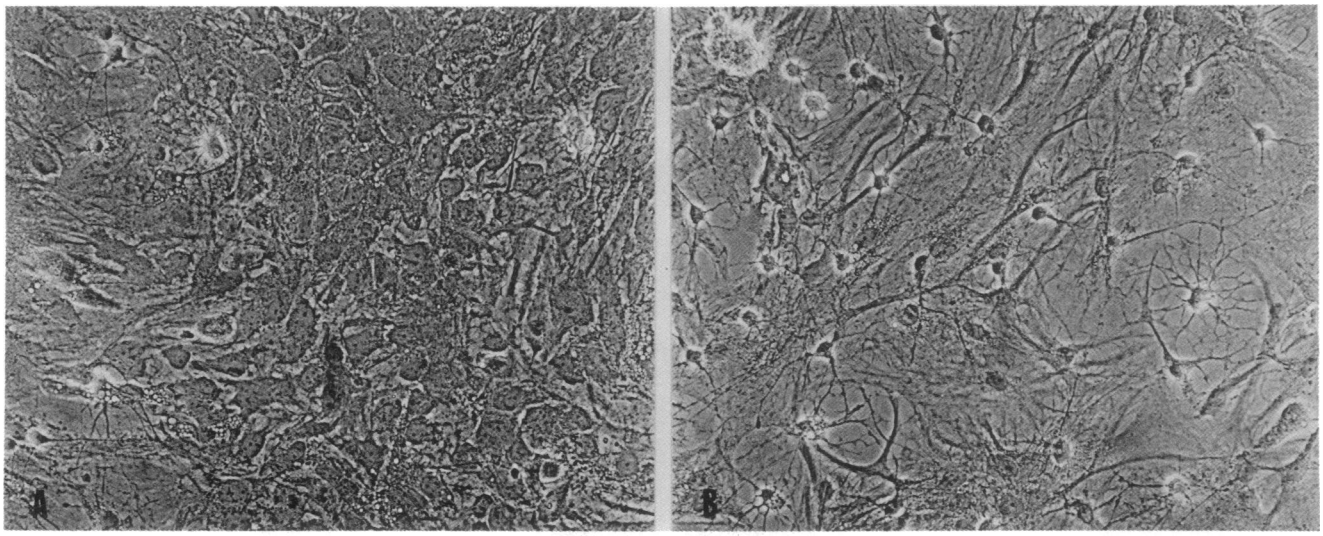

FIG. 5. Phase-contrast micrographs of astrocyte cultures grown for 5 days in serum-supplemented medium (*A*) and CD medium (*B*). (×190.)

been associated with neural tissue or had proven efficacious in other SF media.

A significant difference between our CD medium and others is the lack of necessity for transferrin. Transferrin has been reported to be a stringent requirement for growth of cells in CD media (2). We have tested transferrin over a thousandfold range (1–1000 μg/ml) in CD medium with no observable effect on cell growth. The reason for this difference is not presently understood.

Prostaglandins, including $PGF_{2\alpha}$, are present in the central nervous system and appear to be synthesized at both neuronal and nonneuronal sites (17). We find that $PGF_{2\alpha}$ has a marked stimulatory effect on astrocyte proliferation. Because the rate of release of prostaglandins correlates with the level of neuronal activity (17), this may be one mechanism by which neurons regulate the proliferation of glia during gliogenesis.

The diamine putrescine is a precursor of the polyamines spermidine and spermine. These ubiquitous molecules appear to be involved in growth control in several microorganisms and in mammalian cells (18). More recently, putrescine has been shown to stimulate the proliferation of neuroblastoma cells in culture (7). Although the polyamine content of nervous tissue is known to change in response to nerve injury or to tumor growth, the relationship of these changes to normal neural function remains to be elucidated (19). We find that putrescine has a growth stimulatory effect on normal astrocytes.

Table 1. Effect of culture conditions on attachment of astrocytes at 24 hr

Condition	Total cells attached	
	No. × 10^{-4}	%
Serum-supplemented medium	6.95 ± 0.53	100
SFM	2.44 ± 0.50	35
SFM + fibronectin	4.24 ± 0.28	61
CDM	3.95 ± 0.50	57
CDM + fibronectin	5.80 ± 0.09	83

Purified cultures of astrocytes were trypsinized and washed three times in SF medium (SFM) in order to remove serum proteins. Cells (5×10^4) were plated directly into 35-mm dishes containing either SFM, SFM plus fibronectin (5 μg/ml), serum-supplemented medium (10%), CD medium (CDM), or CDM plus fibronectin (5 μg/ml). Fibronectin (stored in 1 M urea at 4°C) was added to the dishes 15 min before adding the cells. The dishes were tipped in various directions to ensure even coating of the plating surface. At 24 hr, cells were washed, trypsinized, and counted in a Royco 927 TC cell tissue counter and the percentage of the plated cells found attached was calculated. Data expressed is normalized with respect to serum-supplemented medium.

The exact role of insulin in the central nervous system is unknown. Insulin receptors have been identified in the central nervous system by radioligand binding (20–24), although their localization among neural cell types is still controversial. In addition, insulin levels in different brain regions can be 10–100 times higher than plasma levels (25). We are now able to demonstrate that insulin has a growth-promoting effect on purified cultures of astrocytes, though the optimal insulin concentration exceeds physiological values. The high concentration of insulin necessary in our system may be due to an inhibitory contaminant present in the insulin preparation, inactivation of insulin in the medium (26), or cross-stimulation of somatomedin receptors (27, 28). We have also tested a highly purified preparation of insulin obtained from Eli Lilly. The dose–response curve was decreased by 1 order of magnitude, such that the optimal concentration required in this system was now only 5 μg/ml (data not shown). In addition, we observed a stimulatory effect on cell growth with as little as 20 ng/ml.

The requirement for hydrocortisone as a growth-promoting agent in defined medium has been demonstrated for a number of different cell types (29–32). We have encountered great variability in assessing the dose–response characteristics of this hormone in astrocyte cultures. However, 50 nM hydrocortisone provides optimal cell growth in the presence of other supplements.

FGF is a mitogen purified from the pituitary gland or the brain (33). Westermark has reported that FGF is mitogenic for a human glial cell line (34), and Wolfe *et al.* (5) find that FGF has a minor growth-promoting effect on a glioma cell line. We have shown that, by itself, FGF has a small effect on growth, but in the presence of the other factors, it has a marked effect on growth.

In addition to the supplements discussed above, we tested a number of other compounds for their ability to promote astrocyte growth. The following compounds were not observed to be growth-promoting in serum-limited medium (2.5%): thyroxine (10 nM), progesterone (20 nM), testosterone (20 nM), multiplication-stimulating activity (100 ng/ml), and prolactin (100 ng/ml). Fetuin (1 mg/ml) and prostaglandin E_1 (100 ng/ml) strongly inhibited cell growth by 62% and 33%, respectively.

Although growth and viability are good in CD medium, there is still a limitation to consider in the present methodology. We

believe that the serum preincubation for 18–20 hr might only be necessary for plating attachment, because attachment 24 hr after plating directly in CD medium is only 57% of that found for cells plated directly in medium supplemented with 10% fetal calf serum. Furthermore, cells preincubated with medium containing 10% fetal calf serum and then switched to SF medium do not proliferate. This suggests that the washing of the cell cultures effectively removes any serum factors necessary for sustained cell growth. Moreover, we have shown that attachment can almost be fully restored to levels obtained with 10% fetal calf serum by precoating culture dishes with fibronectin. Although we have not yet characterized the effect of fibronectin on the growth kinetics of astrocytes in CD medium, it is now possible to plate directly in CD medium by utilizing fibronectin as a substrate. The increased attachment due to fibronectin was the same regardless of whether the cells were plated in SF or CD medium. This suggests that the supplements in CD medium do not alter the mechanisms by which fibronectin influences attachment *in vitro*. The supplements did have an effect on attachment, however, when used independently of fibronectin. This may be due in part to an increase in cell viability or stimulation of endogenous matrix proteins normally produced by astrocytes for adhesion purposes.

There are obvious advantages to be gained from using a CD medium. Serum is a complex biological fluid which has served as a major supplement for cells *in vitro*. Serum contains growth-promoting and -inhibitory substances which are largely undefined and variable from one batch to another. The replacement of serum by specific growth factors, hormones, and other supplements eliminates this problem. With an established CD medium for pure primary cultures of astrocytes, the regulation of proliferation and differentiation of normal glial cells and the pathological phenomenon of gliosis can be unambiguously analyzed.

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