Continuous Culture of Rat C6 Glioma in Serum-free Medium

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ABSTRACT In this communication we describe serum-free culture conditions for the serial propagation of the C6 glioma cell line. The growth rate, saturation density, and morphology of these cells are equivalent to those of their serum-grown counterparts when cultured in a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's medium F-12 supplemented with trace elements, insulin, transferrin, fibroblast growth factor, linoleic acid complexed to fatty acid-free bovine serum albumin, and a serum-spreading factor (SSF) partially purified from human plasma. The requirement for SSF in the medium can be satisfied by preincubating the tissue culture dishes with SSF. Tissue culture dishes sequentially pretreated with poly-D-lysine and purified cold insoluble globulin will also substitute for this requirement. The fatty acid-free bovine serum albumin/linoleic acid complex increases the growth rate of these cells but has no appreciable effect on their morphology, saturation density, or ability to grow with repeated subculture. The growth stimulation caused by this complex appears to be dependent on the fatty acid, as the fatty acid-free bovine serum albumin alone has no effect on the growth rate. Linoleic acid is cytotoxic in the absence of bovine serum albumin, and the fatty acid-free bovine serum albumin prevents this toxicity. Other fatty acids including oleic, arachidonic, and palmitic only partially substitute for the growth-promoting effect of linoleic acid.

The biochemical and physiological properties of cells are influenced by interactions between the cells and their external milieu. In tissue culture this environment usually consists of a synthetic medium to which has been added crude serum—a complex mixture of molecules, several of which are required for proliferation of the cells. The complex undefined nature of serum as well as the well-known variability between batches of serum may significantly affect experimental reproducibility and, in some instances, the actual experimental result. In addition, most cell types, particularly those of the central nervous system, are not exposed to serum in the intact animal. Thus, at least in this respect, the "normality" of these culture systems relative to normal cells in situ is either clearly lacking or far from certain.

The development of serum-free culture conditions for the growth and maintenance of several established cell lines has been the focus of several recent investigations (1). Taken together, the results from these investigations indicate that the amount of serum needed for the continuous growth of cells can be substantially reduced and, in numerous cases, completely obviated by (*a*) improvements in the low molecular weight portion of the culture medium, (*b*) addition of specific macromolecular growth factors, many having hormonelike properties, and (*c*) modifications of the culture surfaces making them more amenable to cellular attachment, spreading, and growth.

In several of the above-mentioned studies, one or more of the properties characteristic of the particular cell type under study were compared in cells growing in the serum-free and serum-supplemented conditions. In most cases, the behavior of cells in serum-free culture were quite similar to those of their serum-grown counterparts (1). However, in a few instances cells cultured in the absence of serum exhibited biochemical features different from those of cells cultured in serum-supplemented medium. In one such instance, the binding kinetics of radiolabeled epidermal growth factor (EGF) to HeLa cells cultured in the presence of serum were found to be different from those of cultures not exposed to serum (21). Another example has been provided by 3T3-L_1 cells. When the growth of these cells in serum-supplemented medium was arrested at confluence, the cells began to round up and started to accumulate lipid droplets, but when these cells were cultured in serum-free medium this did not occur (18).

The C6 glioma cell line was originally derived from an *N*-nitrosomethyl-urea-induced rat brain tumor (3). C6BU-1, a bromodeoxyuridine-resistant subclone resembling both morphologically and physiologically the cells of origin (9, 5), has been one of the most extensively studied sublines of C6 because of the unique properties of the somatic cell hybrids formed in

THE JOURNAL OF CELL BIOLOGY · VOLUME 87 NOVEMBER 1980 434-441

experiments employing these glioma cells as one of the parents (17). These C6 cell lines have served as a model for the in vitro investigation of glial properties for many years, including the expression of 2':3'-cyclic nucleotide 3'-phosphohydrolase and its regulation by cell density and serum withdrawal (13), the induction of glycerol phosphate dehydrogenase by glucocorticoid hormones (4), and the synthesis of brain specific S-100 protein (3) and glial fibrillary acidic protein (11). In these cells the metabolism of adenosine 3'-5'-cyclic monophosphate (cAMP) has been shown to be hormonally regulated, and alterations in the intracellular cAMP concentration have paralleled marked changes in cellular physiology. Incubation with prostaglandin E_1, β-adrenergic agonists, or theophylline increased the intracellular concentration of cAMP in these cultures (9), and β-agonists, theophylline, or dibutyryl cAMP induced the synthesis of ornithine decarboxylase and lactate dehydrogenase (15). Exposure to dibutyryl cAMP has also been shown to cause a striking alteration in the morphology of these cultured glial cells that was characterized by an increase in the length and number of processes (5).

To simplify the study of the nature and control of the above processes, a serum-free culture system for glial cells in which the cells retain the ability to respond to regulatory hormones, expressed their biochemical markers, and exhibit growth characteristics such that a reasonable number of cells can be obtained for biochemical analysis has been sought for many years. To this end, C6 cells have been adapted to grow in the absence of serum (6, 7). In the examples cited, the cells retained the ability to synthesize S-100 protein and, in one case, even exhibited "morphological differentiation." However, the cells divided with an extended generation time (50–100 h) and either rarely grew to confluence (7) or grew to confluence only when seeded at relatively high densities (6). Moreover, this approach to serum-free culture has the added disadvantage in that it supplies one with little or no useful information on the nutritional and hormonal growth requirements of these cells that could be used to establish new glial cell lines from primary tissue without the necessity of first adapting the cells to culture in serum-supplemented medium.

In the present study we were particularly interested in developing serum-free growth conditions for the C6 glioma that did not require any further adaptation of the cells. The C6 cell line, like most cell lines, was initially established (adapted for growth) in a serum-supplemented medium. We have developed conditions that not only allow the cells to be subcultured from the serum-supplemented condition directly into the serum-free condition without a lag or adaptation period, but also support the continuous growth of C6 glioma cells at rates and saturation densities equivalent to those seen in serum-supplemented medium.

MATERIALS AND METHODS

Cell Stocks

A bromodeoxyuridine-resistant subclone of the C6 cell line, C6BU-1, was obtained from Marshal Nirenberg, National Institutes of Health, Bethesda, Md. The cells were maintained in Dulbecco's modified Eagle's medium (DME) containing 1.2 gm/liter sodium bicarbonate, 25 mM HEPES, 190 IU/ml penicillin, 0.2 mg/ml streptomycin, 25 μg/ml ampicillin, and 10% fetal bovine serum (FBS). The cells were subcultured every 7 d with a split ratio of 1:400.

Experimental Nutrient Medium

In all experiments, a freshly prepared mixture of 3 parts DME to 1 part Ham's F-12 containing 1.2 gm/liter sodium bicarbonate, 25 mM HEPES, 190 IU/ml penicillin, 0.2 mg/ml streptomycin, 25 μg/ml ampicillin, and trace elements (0.5 nM $MnCl_2 \cdot 4\ H_2O$, 0.5 nM $(NH_4)Mo_4O_{24} \cdot 4\ H_2O$, 0.25 nM $NiSO_4 \cdot H_2O$, 15 nM H_2SeO_3, 25 nM $Na_2SiO_3 \cdot H_2O$, 0.25 nM $SnCl_2$, 25 nM $Na_3VO_4 \cdot 4\ H_2O$, and 5 nM $CdSO_4$) was employed as the synthetic nutrient medium (3 DME:1 F12(+TE)), unless otherwise indicated.

Standard Growth Assay

Exponentially growing cells from stock cultures were harvested at 4°C with 0.1% trypsin–0.9 mM EDTA in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS), suspended in 10 ml of 3 DME:1 F12(+TE) containing 0.2 mg/ml soybean trypsin inhibitor, centrifuged, and the pellet was suspended in 3 DME:1 F12(+TE) at 37°C unless otherwise noted.

2 ml of this suspension containing 2.5×10^4 cells were added to replicate tissue culture dishes (35 mm, Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). The indicated experimental additions were made to these dishes from sterile stock solutions, and the dishes were immediately placed at 37°C in a humidified atmosphere containing 5% CO_2. Unless otherwise indicated, the cell number was determined after 5 d of growth with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.), model Z_f. In this procedure, only the cells that were attached to the culture dish were counted, because in all cases, <1% of the cells were unattached.

Preparation and Storage of Factors

All solutions were stored in polypropylene tubes, and aliquoted in volumes such that the solutions were never frozen more than 3 times. The solutions were thawed at room temperature and kept in an ice bath while in use.

The fatty acids were dissolved in ethanol at 250 mg/ml, gassed with nitrogen, and stored at −20°C. Just before use, the fatty acids were diluted with ethanol to a maximal concentration of 25 mg/ml and then added directly to the experimental medium. The concentrations of the fatty acid solutions were adjusted such that each dish received 1–10 μl of solution. Alternatively, the fatty acids were stored for at least 10 wk at 4°C in a PBS solution containing 50 mg/ml fatty acid-free bovine serum albumin (FAF-BSA) and 250 μg/ml fatty acid. To prevent micelle formation the fatty acid stock had to be diluted with ethanol to 25 mg/ml before the preparation of this solution.

Insulin was dissolved in a solution of PBS containing 0.05 M HCl at 6.25 mg/ml and filter sterilized. The solution was stable for at least 8 wk at 4°C.

Transferrin and fibroblast growth factor (FGF) stocks were prepared in PBS at 12.5 mg/ml and 2 μg/ml, respectively. The solutions were filter sterilized, aliquoted, and stored at −20°C.

Human plasma fibronectin (cold insoluble globulin [CIg]) was used and stored as suggested by Collaborative Research Inc. (Waltham, Mass.).

A solution of poly-D-lysine at 1 mg/ml was filter sterilized and stored at −20°C, and diluted just before use.

Serum spreading factor (SSF) preparations were obtained from Dr. David Barnes, University of Pittsburgh. SSF was prepared by a modification of the method of Holmes (2, 10). Outdated human plasma was dialyzed against 0.8% NaCl overnight and clotted by the addition of 0.1% $CaCl_2$. The clot was removed and the serum adjusted to pH 8.0 with 10 N NaOH. This serum was applied to a soda-lime glass bead column (2.5 × 40 cm) previously equilibrated with 0.6 M sodium bicarbonate at pH 8.0 and the column was subsequently washed with 3 bed volumes of 0.6 M sodium bicarbonate at pH 8.0, and 3 bed volumes of H_2O. The adsorbed SSF was eluted with a solution of 0.6 M potassium bicarbonate and 0.2 M potassium carbonate at pH 9.7. Active fractions were pooled, adjusted to pH 7.4, filter sterilized, and frozen. Upon thawing, the active precipitate was dissolved in 10 mM KOH and stored at −20°C. SDS gel electrophoresis of this preparation indicated that the major portion of the protein consisted of several bands with mobilities consistent with mol wt between 60,000 and 90,000. Some protein with greater mobility was also evident. The spreading factor preparation appeared to be free of CIg by electrophoretic analysis (2). In addition, further purification of the spreading factor used in the experiments presented here has produced a preparation in which the activity appears to migrate as a single band on SDS gel electrophoresis with an apparent mol wt between 70,000 and 80,000. The specific activity of this preparation is at least comparable to that of the crude material used to gather the data presented here.[1]

Materials

Bovine insulin, human transferrin, soybean trypsin inhibitor, poly-D-lysine, linoleic acid, arachidonic acid, and oleic acid were from Sigma Chemical Co. (St. Louis, Mo.). Powdered formulations of DME (Cat. No. 430-2100) and F-12 (Cat.

[1] Dr. David Barnes, Department of Biological Sciences, University of Pittsburgh, personal communication.

No. 430-1700) were purchased from Grand Island Biological Co. (Grand Island, N. Y.), FBS from Reheis Co., Inc. (Phoenix, Ariz.), FAF-BSA from Miles Laboratories, Inc. (Elkhart, Ind.), palmitic acid from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.), and purified FGF and CIg were from Collaborative Research.

RESULTS

Serum-free Growth of C6BU-1 Cells

The serum requirement for the continuous growth of C6BU-1 cells in a nutritionally complete synthetic medium can be replaced by a mixture of insulin, transferrin, FGF, FAF-BSA, linoleic acid, and SSF partially purified from human serum (see Materials and Methods). As shown by the growth curve in Fig. 1, C6BU-1 cells plated into 3 DME:1 F12(+TE) supplemented with the above mixture of molecules grew exponentially with a mean generation time of 17.6 h and attained a density after 7 d of growth of 4.0×10^5 cells/cm^2. Both of these growth parameters were almost identical to those measured for their serum-grown counterparts, which divided logarithmically with a mean generation time of 17.2 h before reaching a cell density of 4.1×10^5 cells/cm^2 after the same 7-d period. Furthermore, there was no lag period in the growth of the cells transferred from serum-containing medium into serum-free medium, indicating that no selection or adaptation of the cells had occurred. In addition, the C6BU-1 cells were subcultured twice in this serum-free medium without any appreciable reduction in the growth rate or saturation density (results not shown).

The requirement for SSF in the serum-free medium could be satisfied by simply preincubating the plastic tissue culture surface with medium supplemented with SSF. As shown in Fig. 1, C6BU-1 cells suspended in 3 DME:1 F12(+TE) containing insulin, transferrin, FGF, FAF-BSA, and linoleic acid were plated into tissue culture dishes that had been incubated (24 h, 37°C) with 2 ml of medium supplemented with SSF at 4 μg/ml and thoroughly washed before seeding the cells. In this condition the cells divided at an identical rate and reached a similar saturation density as the cells plated in the presence of a similar concentration of SSF. Therefore, the tissue culture substratum was modified in some manner by the pretreatment with SSF to yield a surface upon which the growth characteristics of C6 cells were similar to those observed in SSF-supplemented medium. Thus, the growth-promoting effects of SSF on C6 cells are probably unrelated to the presence of a contaminating diffusible growth factor, and only the components of crude SSF that are involved in this modification of the tissue culture substratum are important in the elicitation of the growth response.

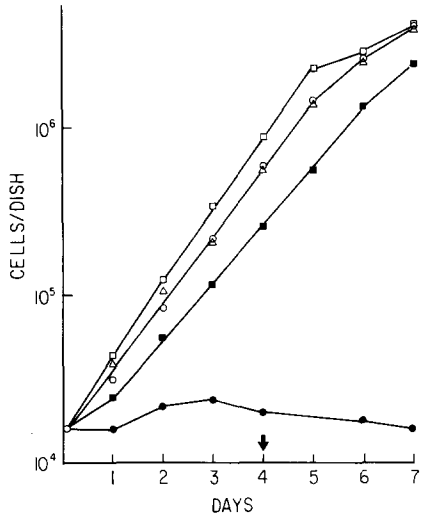

FIGURE 1 Growth curves for C6BU-1. Cells (2.5×10^4/35-mm dish) were plated in 3 DME:1 F12(+TE) (●), 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, and SSF (■), 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, SSF, FAF-BSA and linoleic acid (○), 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, FAF-BSA, and linoleic acid utilizing SSF precoated dishes (△), and 3 DME:1 F12(+TE) supplemented with 5% FBS (□). The concentrations of insulin, transferrin, FGF, SSF, FAF-BSA, and linoleic acid were 25 μg/ml, 25 μg/ml, 50 ng/ml, 4 μg/ml, 1 mg/ml, and 5 μg/ml, respectively. SSF-coated dishes were prepared by incubating dishes with 2 ml of 3 DME:1 F12(+TE) supplemented with SSF at 4 μg/ml for 24 h at 37°C, and then thoroughly washing with medium before seeding the cells. The day 0 point represents the average number of attached cells 3 hours after plating, and this value was determined to be independent of the medium supplements. The arrow indicates a medium change. Values are expressed as the mean of duplicate determinations.

The removal of FAF-BSA and linoleic acid[2] from the serum-free culture medium resulted in a modest increase in the generation time of C6BU-1 cells from 17 to 21 h but had little or no effect on the final attainable cell density (Fig. 1). Moreover, in the absence of FAF-BSA and linoleic acid, C6BU-1 cells appeared morphologically similar to those in FAF-BSA and linoleic acid-supplemented medium, and could be grown, with repeated subculture, for at least 20 cell generations (data not shown).

Effects of Fatty Acids and FAF-BSA

The growth response to linoleic acid, at a constant medium FAF-BSA concentration of 1 mg/ml, is shown in Fig. 2. As shown, the response to linoleic acid increased with increasing concentration up to 5 μg/ml corresponding to a molar linoleic acid to FAF-BSA ratio of 1. Linoleic acid concentrations above this, up to a molar ratio of 2, were growth stimulatory, but the growth response was reduced.

In the same experiment (Fig. 2), the saturated fatty acid, palmitic, the nonessential unsaturated fatty acid, oleic, and the essential unsaturated fatty acid, arachidonic, were also tested for their growth promoting effects. Palmitic and arachidonic acids promoted the growth of C6BU-1 cells to a limited extent, and the effective concentration range was narrow and much lower than that for linoleic acid. Oleic acid stimulated growth in a fashion qualitatively similar to that observed for linoleic acid, but like palmitic and arachidonic acids, its maximal growth-stimulating effect was much less than that of linoleic acid. It is interesting to note that mixtures of optimal concentrations of the various fatty acids were, in no case, as active in the growth promotion assay as linoleic acid alone (data not shown).

Doses of linoleic acid found to be maximally active (5 μg/ml) in the presence of FAF-BSA were extremely cytotoxic in its absence. Approximately 0.5 mg/ml of FAF-BSA was required to prevent this cytotoxic effect; maximal protection was found at a concentration of 1 mg/ml, corresponding to a molar

[2] There may be biologically active linoleic acid present in the commercial medium used in these experiments. However, the amount present does not appear to be adequate for the optimal growth of C6 cells, and if some biologically active linoleic acid is contained in the basal nutrient medium, the concentration (≤0.021 μg/ml) was held constant in all the experiments presented here.

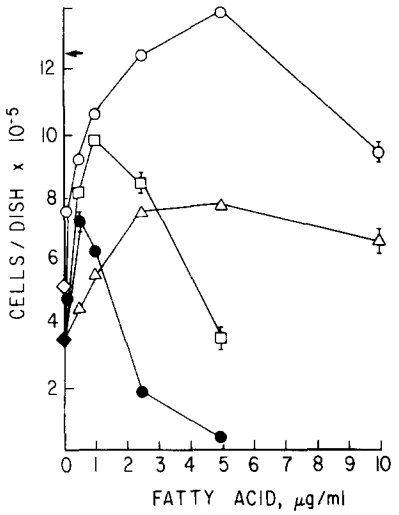

FIGURE 2 Growth response of C6BU-1 cells to individual fatty acids. Cells (2.5 × 10^4/35-mm dish) were plated into 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, and SSF (◇), 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, SSF, and FAF-BSA (◆), and 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, SSF, FAF-BSA, and varying concentrations of linoleic acid (○), arachidonic acid (□), oleic acid (△), and palmitic acid (●). Insulin (25 μg/ml), transferrin (25 μg/ml), FGF (50 ng/ml), SSF (4 μg/ml), and FAF-BSA (1 mg/ml) were used in this experiment. Cell counts were performed on day 5. The arrow indicates the cell number in 3 DME:1 F12(+TE) supplemented with 5% FBS. Data points represent the average of duplicate determinations.

linoleic acid to FAF-BSA ratio of ~1. The cytotoxicity of linoleic acid appears to be related, at least in part, to its instability in aqueous solution when not in association with albumin. Linoleic acid when stored complexed to FAF-BSA in PBS at 4°C quantitatively retained its growth-promoting activity for at least 10 wk; linoleic acid prepared at a similar concentration in PBS alone was cytotoxic after a few days' storage at 4°C, even when added to cells in the presence of FAF-BSA.

FAF-BSA had no growth-promoting activity in the absence of fatty acids and, in fact, slightly inhibited growth under these conditions (Fig. 2). Other evidence suggests that the growth stimulation observed in cultures supplemented with fatty acids and FAF-BSA was primarily caused by the presence of the fatty acid. However, at this time the presence of an active contaminant in the commercial FAF-BSA preparation used in all these experiments cannot be completely ruled out. As illustrated in Fig. 2, the growth response in the presence of 1 mg/ml of FAF-BSA varied with the fatty acid. Furthermore, results equivalent to those shown in Fig. 2 were obtained when the molar fatty acid to albumin ratio was held constant at ~1 (0.5%-wt/wt fatty acid/FAF-albumin, data not shown). In addition, when a small amount of linoleic acid (0.1 μg/ml) was added in the absence of FAF-BSA, no cytolysis was evident, and growth was similar to that observed in cultures that had been supplemented with FAF-BSA and an identical concentration of linoleic acid. Alternative methods for introducing the fatty acids into the culture system have, as yet, been only partially successful (unpublished results), and thus the resolution of the question as to whether the only role of FAF-BSA in this culture system is to function as a fatty acid carrier will have to wait for future experimental verification.

Effects of Modifying the Substratum

SSF influences both the morphology and growth of C6BU-1 cells in serum-free culture medium. As shown by the photomicrographs in Fig. 3, the appearance of cells after 5 d in medium supplemented with insulin, transferrin, FGF, FAF-BSA, and linoleic acid (panel *C*) was strikingly different from that of cells cultured an identical period of time in medium supplemented with insulin, transferrin, FGF, FAF-BSA, linoleic acid, and SSF (panel *D*). In the absence of SSF, the cells grew adhered to the tissue culture plastic substratum, but few, if any, of the cells were flat or extremely extended. If SSF-coated dishes were used, or if SSF was present in the medium, the majority of the cells grew attached to and spread on the plastic tissue culture surface and exhibited the flattened irregular shape typical of their serum-grown counterparts.

To determine whether conditioning of the tissue culture surface with other agents known to promote cell attachment and spreading (14, 22) had a similar affect on C6BU-1 cell growth, dispersed cells were added in medium supplemented with insulin, transferrin, and FGF to tissue culture dishes that were pretreated with either poly-D-lysine, CIg, or poly-D-lysine followed by CIg, and the number of cells per dish was determined 5 d later (Table I). Cells were also plated into dishes that were pretreated with either unfractionated serum, SSF, or poly-D-lysine succeeded by serum or SSF to serve as controls.

Decreasing the net negative charge on the polystyrene by coating with the positively charged polymer, poly-D-lysine, resulted in a small but significant increase in cell number over that in untreated plastic dishes. CIg-treated dishes were no better than untreated dishes in promoting the growth of the cells. However, dishes that had been treated sequentially with poly-D-lysine and then CIg supported growth to an extent greater than either treatment alone, and the cell number at the end of 5 d was, in this case, ~70% of that in control cultures. SSF- and FBS-pretreated dishes were equally effective in supporting growth and, in each case, the prior treatment with poly-D-lysine had little or no additional effect.

The growth of C6BU-1 cells in medium supplemented with insulin, transferrin, and FGF was enhanced by SSF in a concentration-dependent fashion (Fig. 4 *C*). In the absence of SSF, the number of cells per dish increased from 2.5 × 10^4 (the number of cells initially plated) to 1.6 × 10^5 over a 5-d incubation period, representing two to three cell doublings. When SSF (4 μg/ml) was added to the culture medium at the time of subculture, the cells divided an additional two times attaining a density of 6.1 × 10^5 cells/dish. Increasing the concentration of SSF in the medium above 4 μg/ml caused a decline in the growth response; and this decline was paralleled by a change in cell shape from a spread configuration to a spherical one (unpublished observation). In a similar experiment, the growth effects of SSF-coated dishes varied, in a linear fashion, with the concentration of SSF present in the preincubation medium used to coat the tissue culture dishes (concentrations >4 μg/ml were not assayed).

Effects of Insulin, Transferrin, and FGF

The deletion of insulin, transferrin, or to a lesser extent, FGF from a mixture of insulin, transferrin, FGF, and SSF significantly reduced the growth response compared to that when all four supplements were added simultaneously (compare in Fig. 4 *A*, *B*, and *D*, the growth response at zero concentration and

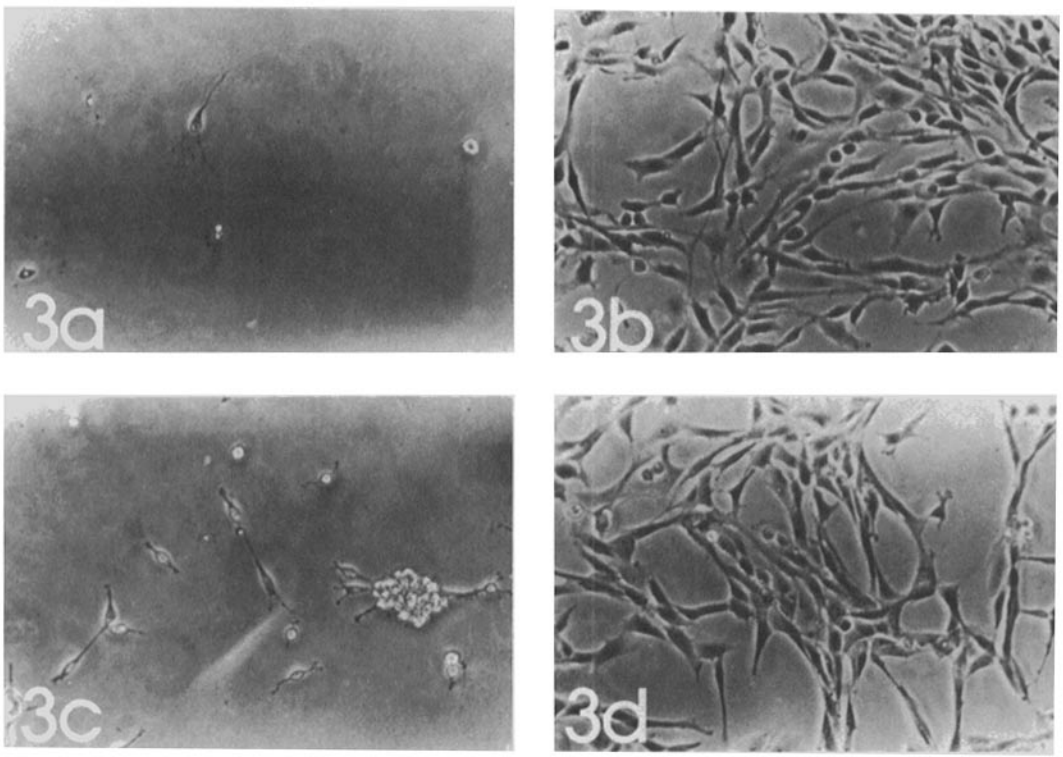

FIGURE 3 Photomicrographs (all phase contrast × 150) of C6BU-1 cultured for 4 d in (*a*) 3 DME:1 F12(+TE), (*b*) 3 DME:1 F12(+TE) supplemented with 5% FBS, (*c*) 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, FAF-BSA, and linoleic acid, and (*d*) 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, FAF-BSA, linoleic acid, and SSF. Insulin (25 μg/ml), transferrin (25 μg/ml), FGF (50 ng/ml), FAF-BSA (1 mg/ml), linoleic acid (5 μg/ml), and SSF (4 μg/ml) were used in this experiment.

TABLE I

Effects of Modifying the Substratum on C6BU-1 Cell Growth in Serum-free Supplemented Medium

Solution used to pre-pretreat dishes (2 ml, 5 min, 25°C)	Solution used to pretreat dishes (2 ml, 24 h, 37°C)	Cell No. × 10^{-5}	Relative cell No.
None	None	1.57 ± 0.12	1.0
Poly-D-lysine, *0.1 mg/ml*	None	2.91 ± 0.12	1.9
None	5% (vol/vol) FBS	6.02 ± 0.21	3.8
Poly-D-lysine, *0.1 mg/ml*	5% (vol/vol) FBS	5.89 ± 0.04	3.8
None	Clg (5 μg/ml)	1.60 ± 0.02	1.0
Poly-D-lysine, *0.1 mg/ml*	Clg (5 μg/ml)	4.21 ± 0.24	2.7
None	SSF (4 μg/ml)	6.10 ± 0.18	3.9
Poly-D-lysine, *0.1 mg/ml*	SSF (4 μg/ml)	5.27 ± 0.11	3.4

Cells (2.5×10^4, 35-mm dish) were seeded in the presence of insulin (25 μg/ml), transferrin (25 μg/ml), and FGF (50 ng/ml). All dishes were washed twice with 2 ml 3 DME:1 F12(+TE) before seeding cells. Poly-D-lysine-treated dishes were washed twice with 2 ml H_2O before subsequent treatment. Cell number was determined 5 d after plating. Values represent the average (±SD) of triplicate determinations.

that at the maximally effective concentration). The concentration of insulin, transferrin, and FGF required for optimal growth was 25 μg/ml and 50 ng/ml, respectively, when determined in the presence of optimal concentrations of the other factors. The concentration dependency of the growth response for each factor (insulin, transferrin, FGF, and SSF) was not altered by the addition of FAF-BSA (1 mg/ml) and linoleic acid (5 μg/ml) to the culture medium (data not shown).

Influence of the Low Molecular Weight Nutrients on Cell Growth

The defined basal medium in the above experiments is a mixture of DME and Ham's medium F-12. DME, which contains relatively high concentrations of the essential amino acids and sugars, is representative of the commercially available media formulated for the mass culture of cells with serum supplementation. Ham's F-12, which was formulated for clonal protein-free growth and is also commercially available, contains low concentrations of the essential amino acids and sugars, and includes lipids, nucleic acid derivatives, vitamins, and nonessential amino acids that are not present in DME. Therefore, an easily obtainable and sufficiently complex basal medium that also contains adequate concentrations of nutrients to support the mass culture of cells in low concentrations of serum can be fabricated by mixing DME and F-12. In fact, this mixture, when supplemented with the appropriate growth factors, supports the serum-free growth of many different cell types (1).

In medium supplemented with 5% (vol/vol) serum or less, C6 cells grew significantly better in a mixture of DME and F-12 than in either formulation alone (results not shown). In addition, both dialyzed and nondialyzed FBS were equally effective in promoting the growth of C6BU-1 cells when tested in a mixture of DME and F-12; this was not true for the same cells in either DME or F-12 alone.[3]

[3] Dr. S. Ohasa, Osaka University, Osaka, Japan, unpublished results.

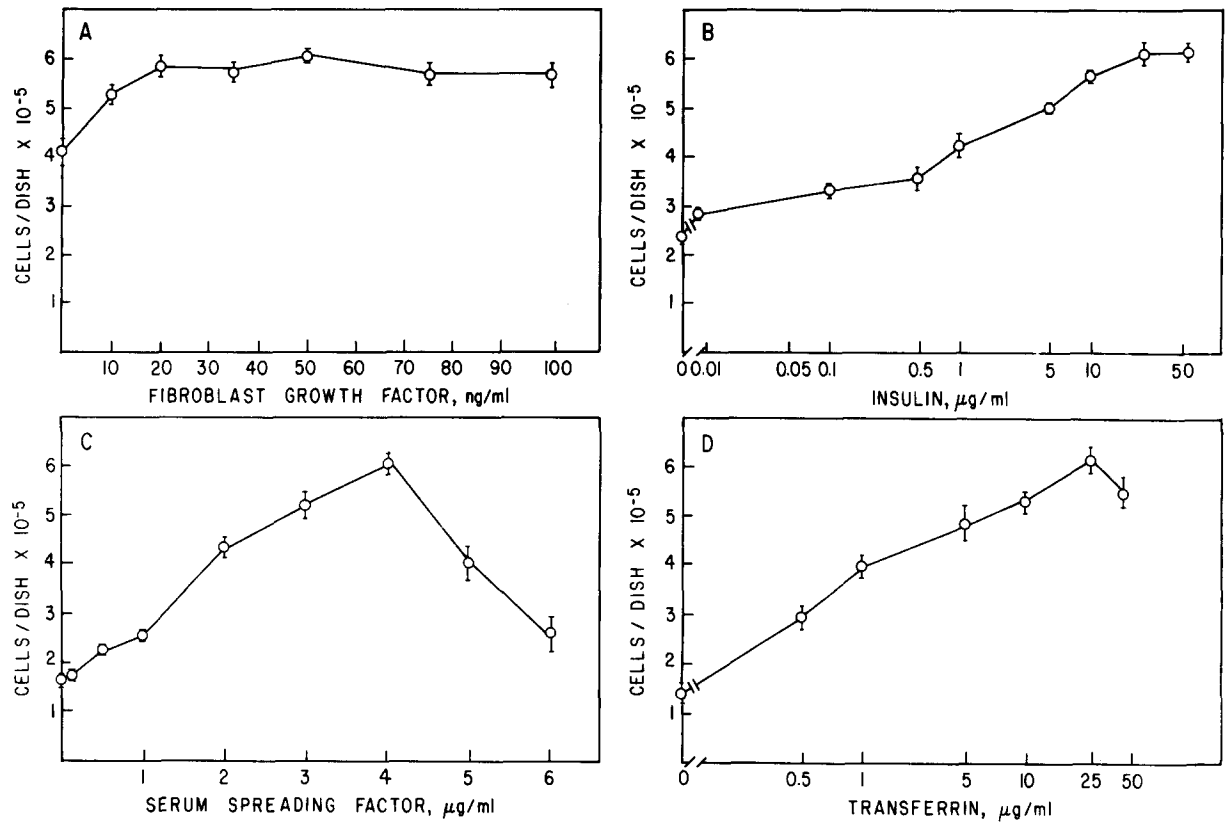

FIGURE 4 Growth response of C6BU-1 cells as a function of FGF (*A*), insulin (*B*), SSF (*C*), and transferrin (*D*) concentration in FAF-BSA, linoleic acid-free 3 DME:1 F12(+TE). In each case the concentration of three of the four supplements was held constant at optimal levels (50 ng/ml FGF, 25 μg/ml insulin, 4 μg/ml SSF, and 25 μg/ml transferrin) while the concentration of the fourth was varied as indicated. The cell number was determined on day 5, and values represent the mean (±SE) of duplicate determinations.

TABLE II

Effect of the Synthetic Portion of the Culture Medium on the Serum-free Growth of C6BU-1 Cells

DME	F-12	Cells/Dish × 10^{-5}
% (vol/vol)		
0	100	3.04 ± 0.16
50	50	10.01 ± 0.01
75	25	13.64 ± 0.49
83	17	12.92 ± 0.16
100	0	3.79 ± 0.66

Cells (2.5 × 10^4/35-mm dish) were plated into either DME, F-12, or mixtures of DME and F-12 prepared at the indicated ratios and cell growth was determined 5 d later. All dishes contained HEPES (25 mM), sodium bicarbonate (1.2 gm/l), antibiotics, trace elements, insulin (25 μg/ml), transferrin (25 μg/ml), FGF (50 ng/ml), FAF-BSA (1 mg/ml), linoleic acid (5 μg/ml), and SSF (4 μg/ml). The data is expressed as the mean (±SD) of triplicate cultures.

The effect of varying the synthetic portion of the culture medium on serum-free C6BU-1 cell growth can readily be seen in the following experiment where equal numbers of cells were seeded into DME, F-12, or mixtures of DME and F-12 at varying ratios, and their subsequent growth measured 5 d later (Table II). Although growth in F-12 was slightly better than in DME, the growth observed in both cases was significantly less than that seen in mixtures of DME and F-12. The maximum number of cells per plate was reached in a mixture of three parts DME and one part F-12, and growth in this mixture was approximately fourfold greater than that seen in the DME and F-12 control cultures. Furthermore, the cell number observed in serum-free supplemented 3 DME:1 F12(+TE) was equivalent to that noted for comparable cultures grown in the same medium supplemented with a maximally effective concentration of FBS (5% vol/vol).

DISCUSSION

Heretofore, the only rat C6 glioma cells that were capable of reasonable growth in serum-free tissue culture were adapted to proliferate in this condition. The results of the present study show that C6 cells can be grown continuously in a serum-free environment without selection or adaptation. To accomplish this, the cells require (*a*) a complex synthetic nutrient medium similar to that provided by a 3 part to 1 part mixture of DME and F-12 supplemented with trace elements, (*b*) the polypeptide hormones insulin and FGF, (*c*) the iron transport protein, transferrin, (*d*) linoleic acid bound to bovine serum albumin, and (*e*) a properly modified substratum that can be achieved by either supplementing the culture medium or pretreating plastic tissue culture dishes with SSF. When all these requirements are met, the cells, from a low population density, divide exponentially with a generation time of 17.6 h (compared to 17.2 h in serum-supplemented medium), attain a saturation density of 4.1 × 10^5 cells/cm^2 (compared to 4.0 × 10^5 cells/cm^2 in serum-supplemented medium), retain a morphology similar to that of their serum-grown counterparts, are capable of repeated subculture, and retain their hormone responsiveness.

Although C6 cells grow optimally in serum-free culture only when all of the above requirements are met, it is possible to cultivate these cells for extended periods of time, with little or no effect on the growth rate, saturation density, or morphology, without successfully fulfilling all of these requirements. For

some types of research that directly involve the characterization of the pharmacological/physiological effects on glial cells of molecules that bind to BSA, the BSA can be eliminated from the culture medium provided the concentration of linoleic acid is substantially reduced. In such cases, the cells grow with only a somewhat longer generation time (21 h compared to 17 h) than those in optimally supplemented medium. On the other hand, the relative ease in which the different fatty acids can be varied (in the presence of FaF-BSA) provides one with a simple system for the study of lipid metabolism and the influence of fatty acid source on the fluidity and function of the plasma membrane.

Whenever the experimental objectives require totally defined growth conditions, the FAF-BSA/linoleic acid complex can be eliminated and the polylysine-CIg-modified tissue culture surfaces can be substituted for surfaces pretreated with SSF, which at present is a mixture of a few different proteins. The C6 cells appeared to grow equally well on tissue culture surfaces modified by either of these procedures, with only a slight reduction in the mean generation time with the polylysine-CIg pretreated substratum.

If the expense and availability of materials is of primary importance, tissue culture dishes pretreated with calf serum (as opposed to FCS, SSF, or poly-D-lysine-CIg) provide for reasonable growth without severely altering the relative simplicity of the culture system (only a small fraction of the serum proteins actually adsorbs to tissue culture plastic [8]). In addition, the growth-promoting effects of fibroblast growth factor are relatively minor, and the FGF can possibly be eliminated from the system, or at least reduced to 10 ng/ml.

All of these growth conditions provide for uniformity of medium composition, and the well-known variation seen among serum lots is eliminated. Furthermore, the expense of serum-free culture of C6 cells is not unreasonably high, considering the cost, variability, and recent problem of serum availability that are inherent with serum-supplemented culture systems.

Factors not present in this serum-free culture system that are known to influence cellular physiology were also tested for their effects on the growth of C6 cells. Increasing the intracellular concentration of cAMP by treatment with cholera toxin or dibutyryl cAMP has been reported to stimulate the growth of Schwann cells cultured from rat sciatic nerve (16). Addition to the serum-free culture medium of dibutyryl cAMP or physiological levels of agents known to elevate the intracellular concentration of cAMP such as prostaglandin E_1, antidiuretic hormone, epinephrine, and norepinephrine inhibited the growth of C6BU-1 cells. Secretin, carbamyl choline, dexamethasone, and dibutyryl cGMP were also found to be growth inhibitory. Although EGF has been reported to stimulate the multiplication of normal human glial cells in vitro (20), we did not observe any effects of EGF on the growth of C6 cells. Nerve growth factor, progesterone, testosterone, and estradiol were similarly inactive in this growth assay.

Slightly improved growth was observed in the presence of the mixture of trace elements included in the basal nutrient medium. Each trace element, at the concentration contained in the mixture, was individually tested for its effects on C6BU-1 growth and none was found to be growth inhibitory. These experiments were performed with cells subcultured from serum-supplemented medium, and thus the beneficial effects of the individual elements may not have been obvious for the first few generations of growth that were investigated. While not at the present time widely accepted as an essential trace element, cadmium was included because it has been found to be beneficial for the growth of HeLa cells and GH3 cells (1).

During the course of this work we found that C6 cells exhibited a marked mitogenic response to several partially purified factors only when the cells were cultured under suboptimal growth conditions. This was the case for the preparations of gimmel factor, a protein contained in extracts of female rat submaxillary glands (18) that also promotes the growth of human astrocytoma and 3T3 cells. Recently, it has been demonstrated that hypothalmic extracts contain endothelial cell growth factor activity that can be separated from hypothalamic FGF by Sephadex G-100 gel exclusion chromatography (12), and we found that this factor markedly stimulated the growth of C6 cells.

The growth-promoting effects of these supplements are not unique to one subclone of the C6 line. C6 cells purchased from American Type Culture Collection, and other sublines of C6 were also found to propagate in 3 DME:1 F12(+TE) supplemented with insulin, transferrin, FGF, linoleic acid, FAF-BSA, and SSF. Thus the growth regulation of these various subclones appears to be as consistent as the biochemical, physiological, and pharmacological properties previously reported.

One of the most characterized differentiated properties of the C6 cell line is β-adrenergic receptor-mediated functions. We find that the binding characteristics of ^{125}I-iodohydroxybenzylpindolol (^{125}I-HYP) to the β-adrenergic receptor of these C6 cells are similar in cells cultured in either serum-supplemented media or the serum-free system presented here. This includes the forward rate constant for ^{125}I-HYP binding, the stereospecific displacements of ^{125}I-HYP by β-adrenergic agonists and antagonists, and the linear variation of receptor density with cell density. Preliminary results indicate that maximal binding occurs after a 1-h incubation period at 37°C, and the number of specific binding sites per cell is $1.85 \pm 0.22 \times 10^4$ for serum-free cultures and $1.21 \pm 0.16 \times 10^4$ for cells grown with serum-supplemented medium.[4] These findings are in accordance with previously reported results employing C6 cells maintained in serum-supplemented medium (19), and indicate that this serum-free culture system supports not only the growth but also some differentiated functions of the C6 cell line.

We thank Dr. David Barnes for his critical reading of the manuscript and helpful discussions. This work was supported by National Institute of Health grant U. S. Public Health Service CA 19731.

Received for publication 11 February 1980, and in revised form 28 July 1980.

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