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Synthesis and Secretion of a Nerve Growth-Stimulating Factor by Neonatal Mouse Astrocyte Cells *in Vitro*

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ABSTRACT. Neonatal mouse astroglial cells cultured in a serum-free medium synthesize and secrete a trophic growth factor which resembles nerve growth factor (NGF). The NGF-like factor reacts with antiserum to β subunit of NGF (β-NGF) and, after labeling with [^{35}S] cystine, migrates similarly to purified mouse β-NGF in SDS polyacrylamide gel electrophoresis and Sephadex G-100 gel filtration systems. The astrocyte cell NGF-like factor displays β-NGF-like neurite growth-promoting activity for the clonal rat pheochromocytoma (PC-12) cell line and this bioactivity is blocked by β-NGF antiserum. These results indicate that NGF-like factor synthesized and secreted by astroglial cells, is similar, if not identical, to β-NGF from the mouse submandibular gland and further support a potential role for NGF in the central nervous system. (*Pediatr Res* 20: 367–372, 1986)

Abbreviations

NGF, nerve growth factor
β-NGF, β subunit of NGF
CNS, central nervous system
GCM, glial conditioned medium
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA, trichloroacetic acid

NGF is a protein known to be necessary for the growth and maintenance of sympathetic neurons of the autonomic nervous system, as well as certain sensory neurons of the peripheral nervous system. Recent evidence suggests that there also are significant concentrations of NGF in the CNS. Immunoreactive NGF has been reported in adult brain tissue of several species (1–5), and we have reported finding NGF immunoreactivity in the brain of developing, as well as adult, mice (6). Scott *et al.* (7) reported finding bioactive NGF in homogenates of CNS tissue from adult mice. Since NGF does not cross the blood brain barrier, CNS NGF is thought to be produced locally. However, the possible site of production has not been clearly elucidated.

NGF has been reported to be synthesized by such cell lines and tissues in culture as Sarcoma 37 and 180 (8), glioma (9) and neuroblastoma cells (10), 3T3 and L cells (11), rat skeletal muscle cells (12), human fibroblasts (13), chick dorsal root ganglia (14), primary chick fibroblasts (15), and mouse adrenal medulla (16) and heart (17–20), among others. Although human glial cells in culture have been reported to release an NGF-like substance (21), there has been little additional direct evidence for the synthesis or release of NGF or NGF-like growth factors from normal brain tissue. In the present report, we describe the synthesis and secretion of an immunoreactive and bioactive NGF-like factor with biochemical characteristics of NGF from cultures of cerebral tissue derived from 1- to 2- and 12-day-old mice. Staining, histological, and growth characteristics suggest that the cultures consist predominantly of astrocytes. These data provide the first direct evidence that an NGF-like factor is produced by astrocytes derived from the neonatal mammalian brain.

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MATERIALS AND METHODS

Cell culture. The cerebral hemispheres were rapidly removed from either 1- to 2- or 12-day-old Swiss-Webster mouse pups killed by decapitation and placed into cold Honegger's Saline D1, pH 7.4 (22) until the dissections were complete. The tissue was finely minced with scissors, washed twice with ice cold Honegger's Saline D1, and extracted with 0.25% Viokase (Viobin Corp) in Ham's saline B, pH 7.4, for three 10-min dissociation intervals at 35° C. Each cell extraction was passed through a 37-μ nylon sieve to insure a single cell suspension. The suspensions were collected into 50-ml centrifuge tubes containing resin-treated (23) macromolecular fetal bovine serum protein (FBSP-R) (24) and centrifuged at 800 rpm for 10 min at room temperature. The resultant cell pellet was suspended in Fisher's mouse astrocyte medium (25), modified to contain Delbecco's modified Eagle's medium with 4.5 g/liter glucose supplemented with biotin (10 μg/ml), selenous acid (30 nM), and FBSP-R (1 mg/ml). The final osmolality was 320 mOsmol/liter.

A cell density of 2×10^5/ml was distributed in 10 ml to culture flasks having a surface area of 75 cm^2; the cells were incubated at 37° C in humidified 5% CO_2/air. The cultures were refed on days 3, 6, and 9. On day 9 after feeding, the flasks were placed on a rotary shaker at 35° C for 24 h (250 rpm) to remove over 95% of the contaminating oligodendroglial cells as described by McCarthy *et al.* (26) and Morrison and de Vellis (27). After shaking, the medium containing the oligodendroglial cells was discarded and the cultures were washed twice in Ham's saline B. They were fed G3 glial medium as described by Bottenstein (28) for 3 h, refed again with G3 medium for 5 h; spent medium was discarded. The cultures were fed a third time with G3 medium to which cells were exposed for 5 days. After this time the glial conditioned G3 medium was collected and the cultures were refed with mouse astrocyte medium for 3 more days prior to repeating the conditioning process.

Preparation of GCM. The GCM was prepared from the expended G3 medium by centrifugation at 48,250 × *g* for 1 h at 5° C and dialyzed for 48 h against two changes (200 volumes) of 0.01 M ammonium acetate, pH 5.2, at 4° C in dialysis tubing

with a molecular weight cutoff of 3500. The GCM was lyophilized and stored at −70° C until used. For analysis the GCM was reconstituted in normal saline at 50 mg solids/ml (=3.25 mg protein/ml) and filtered through a 0.22-μ Millex filter (Millipore). Measurement of protein was performed by either of two methods (29, 30).

Bioassays. The bioassay, using the clonal rat pheochromocytoma cell line (PC-12) as described by Green (1), was used with the following exceptions: the plates were not coated with polylysine and the cells were not "primed" with NGF prior to the assay. The PC-12 cells were obtained from Dr. Lloyd Green and maintained in RPMI-1640 medium containing 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum. For the bioassay, both sera were omitted. The cells were harvested from logarithmic-growing cultures, washed three times with Ham's saline B, then resuspended in RPMI-1640 medium at a cell density of 2×10^4 cells/ml. One ml of the cell suspension was added to each well of six well cluster plates (Costar) having a surface area of 8 cm^2. Three doses of GCM were used: 50, 100, and 200 μl (162.5, 325, and 650 μg protein, respectively). Rabbit anti-β-NGF having a titer of 1:150,000, as determined by radioimmunoassay, was diluted 1:10 and added at a single dose of 200 μl (1:1500 final titer) to opposing wells containing the three doses of GCM. The total volume of each well was brought to 2 ml with the addition of RPMI-1640. The plates were incubated at 37° C in a humidified atmosphere of 5 CO_2/air. The bioassay was read at 48 and 72 h and visually quantified as the percentage of neurite-bearing cells in the test wells in comparison to control wells.

[^{35}S] cystine labeling and immunoprecipitation. The labeling and immunoprecipitation were conducted as described by Berger and Shooter (31, 32). Subconfluent cultures in passage two (24 days *in vitro*) were detached with 0.25% trypsin and 0.02% EDTA in Tris buffer, pH 7.8. The trypsin was neutralized with soybean trypsin-inhibitor and the cells harvested by centrifugation at 800 rpm for 10 min. The resultant cell pellet was resuspended in the mouse astrocyte medium to a density of 2×10^5 cells/m. One ml of the cell suspension was added to each well in six well cluster plates. The plates were incubated overnight at 37° C in humidified 5% CO_2/air to effect cell attachment. The plating medium was then aspirated and discarded, the wells were rinsed twice with Ham's saline B, and 2 ml of G3 medium were added to all wells and the plates returned to the incubator for 3 h. The medium was aspirated and discarded. The cells were refed and incubated for a further 5 h after which time they were fed G3 medium containing 626 μg/ml cystine (as carrier) and returned to incubate.

The cultures were labeled at 24, 48, 72, 96, or 120 h after plating by adding 1 μCi [^{35}S] cystine (specific activity 487 Ci/mM, New England Nuclear) to each well, incubating for 10 min at 37° C in 5% CO_2/air, and following with a 150-min pulse chase. At the completion of the pulse chase, 500 μl from each supernatant were spotted on 2.4-cm glass filters (Whatman), precipitated with ice cold 10% TCA, washed with cold ethanol, and dried overnight at 37° C prior to counting. The remaining supernatants were collected into individual polystyrene 12 × 75 mm tubes, flash frozen in dry ice-ethanol, and lyophilized.

The cells were harvested from the wells by scraping with 500 μl lysing homogenization buffer, pH 4.0, and incubated in an ice bath for 10 min to effect lysis. The lysates were transferred to ice cold 1.5 ml microfuge tubes (Beckman), sonicated for 1 min, and centrifuged for 1 h at 48,250 × g at 4° C. The supernatants were removed and 20 μl pretitered rabbit β-NGF antiserum (diluted 1:10) were added to each tube. The tubes were vortexed, incubated in an ice bath for 48 h at 4°C, and centrifuged at 10,000 × g for 5 min at 4° C. The resultant immunoprecipitates were washed with an acetate wash buffer, pH 4.0, dissolved in 250 μl 0.1 N NaOH and then neutralized with 250 μl 0.1 HCl. To each of these tubes 55 μl of incubation buffer (sodium acetate 0.5 M with 10 mg/ml bovine serum albumin, pH 4.0) as a suspension, 20 μl β-NGF antiserum (1:10 dilution), and 30 μl β-NGF (50 μg/ml) as carrier were added. The mixture was incubated for 24 h at 4° C. The immunoprecipitates were collected by centrifugation, washed once with 500 μl wash buffer, and prepared for SDS-PAGE analysis.

The lyophilized supernatants were reconstituted to 250 μl with wash buffer and immunoprecipitated in the same manner as the cell lysates.

SDS-PAGE analysis. This analysis was performed by the method of Laemmli (34). The immunoprecipitates were incubated in 100 μl modified sample buffer, pH 6.8 (Tris-HCl 0.0625 M, SDS 2%, 2-mercaptoethanol 5%, and urea 9 M), at 37° C overnight. The samples were run on acrylamide: bisacrylamide (15:0.375; 80 × 5 mm). Electrophoresis was performed using a Tris (0.025 M), glycine (0.192 M), SDS (0.1%) buffer, pH 8.3, and 4 mA/gel until the dye front ran off the bottom of the gel (6 h). The gels were removed, cut into 2-mm slices, and incubated overnight at 37° C in 8 ml scintillation fluid (POPOP 4 g, POP 0.05 g, 54 ml PROTOSOL from the New England Nuclear, and 6 ml H_2O/liter of toluene). The slices were counted in a multichannel liquid scintillation counter. Control gels containing purified β-NGF for staining and [^{125}I] β-NGF for slicing and counting were run at the same time in the system. The control gels were fixed in 50% TCA overnight, then stained at 37° C for 1 h in freshly prepared Coomassie B (0.1%) in 50% TCA, followed by destaining in 7% acetic acid. The SDS-PAGE analysis of the GCM was performed by destaining in 7% acetic acid. The SDS-PAGE analysis of the GCM was performed using 5–20% linear gradient gels. Fifty μl of the immunoprecipitated GCM were incubated with 50 μl of the modified sample buffer at 90° C for 10 min. Fifty μl were loaded onto each of two of the linear gradient gels prerun for 30 min. Electrophoresis conditions and buffer were as described previously. Control gels contained the following standards 1) phosphorylase, 2) bovine serum albumin, 3) ovalbumin, 4) carbonic anhydrase, 5) soybean trypsin inhibitor, 6) α-lactalbumin, 7) aprotinin, and 8) insulin (β-chain). Mouse β-NGF gels were run at the same time. The gels were fixed, stained, and destained as noted previously.

Gel filtration. This was performed as described by Furukawa *et al.* (20). Five hundred μl of GCM were applied to a 1 × 50 cm Sephadex G-100 (Pharmacia) column preequilibrated with 0.1 M phosphate buffer, pH 7.0, containing 0.3 M NaCl and 1 mM $MgCl_2$. The flow rate was 3 ml/h and 800 μl fractions were collected. The fractions were screened for NGF activity by one site radioimmunoassay as described by Walker *et al.* (33). The fractions containing β-NGF were pooled, lyophilized, and subsequently tested for bioactivity. The column was calibrated for estimation of molecular weight using blue dextran, trypsinogen, and cytochrome C. The column was then flushed and reequilibrated with buffer. [^{125}I] β-NGF (10,000 cpm) was added and the column run as noted previously. The fractions were counted on a γ counter.

RESULTS

The mouse cerebral cells cultured in a modified astrocyte medium assumed the same morphology as similar cells grown in serum containing medium. However, upon subsequent passage they tended to grow as clusters, eventually spreading and flattening out. Growth rates tended to be somewhat slower in the modified medium. Histochemical studies of this cell line showed a glutamine synthetase level equal to that found simultaneously in a rat astrocyte cell line known to be free of meningeal fibroblast contamination. Similarly, histochemical and immunofluorescent studies revealed at least 80% of cells to contain glial fibrillary protein. Considering that newly replicated astrocytes in a developing astrocyte culture may not show a positive reaction for glial fibrillary protein, these findings are highly suggestive that the culture is composed predominantly of astrocytes. In addition, because meningeal cells rarely thrive in the medium described,

and because our attempts to grow postnatal mouse skin fibroblasts in the medium were unsuccessful, we conclude that our cultures are composed predominantly of mouse brain astrocytes. We cannot, however, exclude the possibility of minimal contamination with meningeal cells.

The PC-12 bioassay was performed to determine the neurite promoting and neuronal survival properties of the GCM. A low density culture (1×10^4 cells/well) of rat pheochromocytoma cells (PC-12) was treated with the reconstituted GCM added at 50, 100, and 200 μl (with opposing wells receiving the same doses); a single dose of 200 μl rabbit anti-β-NGF was used to block any NGF-mediated effect. The resultant neurite outgrowth effect with GCM was directly dose dependent. Representative plates with 200 μl GCM from 2- and 12-day astrocyte cultures are shown in Figure 1 *b* and *c*. The effect of purified NGF (50 ng/ml) is shown in Figure 1*a*. The wells containing 50 and 100 μl GCM were completely blocked by the β-NGF antiserum. A representative plate from 2-day astrocytes and 100 μl GCM blocked with antibody is shown in Figure 1*d*; the wells containing 200 μl GCM were not completely blocked.

The kinetics of NGF-LF synthesis and secretion by the astroglial cells were characterized by labeling and immunoprecipitation experiments. After pulse-chase labeling with [^{35}S] cystine at 24, 48, 72, 96, and 120 h (Fig. 2), there was a lag period of about 48 h before significant labeling of NGF-like immunoprecipitable material in cell lysates was observed. Label incorporation increased rapidly through 72 h, plateaued, and fell off rapidly thereafter. Using TCA precipitation, secretion into the medium increased slowly to 96 with maximal levels of medium TCA precipitable label between 96 and 120 h (not shown).

The SDS-PAGE results are shown in Figure 3. A 5–20% linear gradient was utilized. Both the 2- and 12-day GCM (tubes 3 and 4) showed large molecular weight, poorly resolved components of considerable concentration. In addition, however, each showed a faintly staining band migrating similarly to the purified mouse β-NGF standard (tube 2). The β-NGF standard ran near the α-lactalbumin marker (faint band 6, tube 1). The aprotinin and β-chain insulin standards ran off the gels.

The SDS-PAGE (15%) gels run with [^{35}S] cystine labeled GCM, unlabeled β-NGF, and iodinated β-NGF are shown in Figure 4. These three components ran at the same relative front on the gels. Because of low counts with cystine-labeled GCM, only one slice contained sufficient counts above background to be considered.

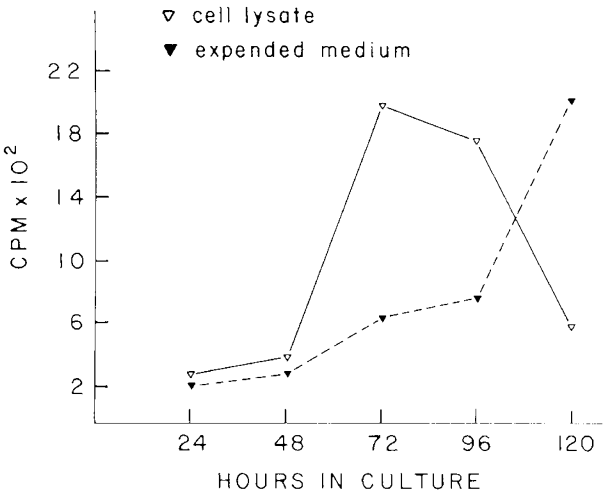

Fig. 2. [^{35}S] cystine pulse-chase cell lysate (△) and expended medium (▲) precipitated with β-NGF antiserum at 24, 48, 72, 96, and 120 h of culture.

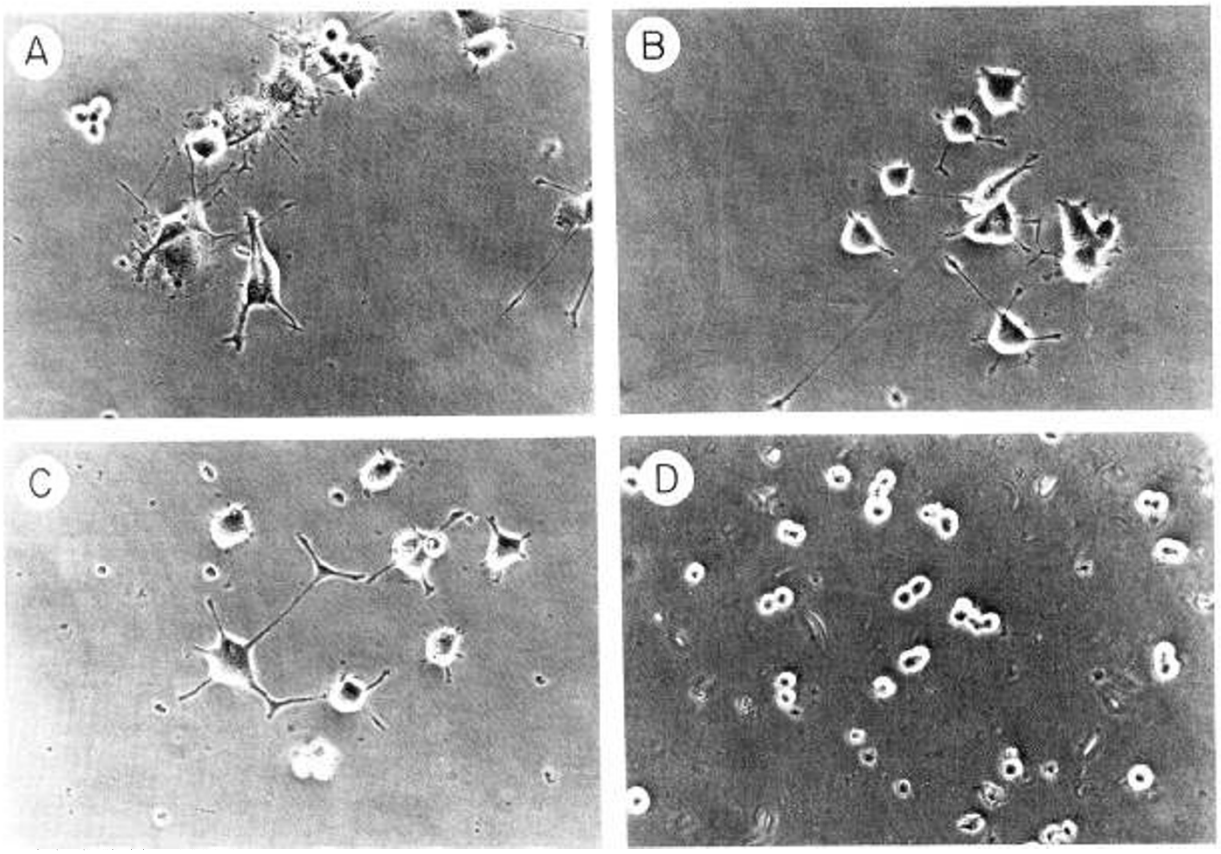

Fig. 1. Bioassay with PC-12 cells plated at a density of 2×10^4 cells/2 ml. *A*, β-NGF, 50 ng/ml; *B*, GCM 2, 200 μl, 650 μg protein/ml; *C*, GCM-12, 200 μl, 650 μg protein/ml; *D*, GCM-2, 100 μl, 325 μg protein/ml + β-NGF antibody. All parts ×200.

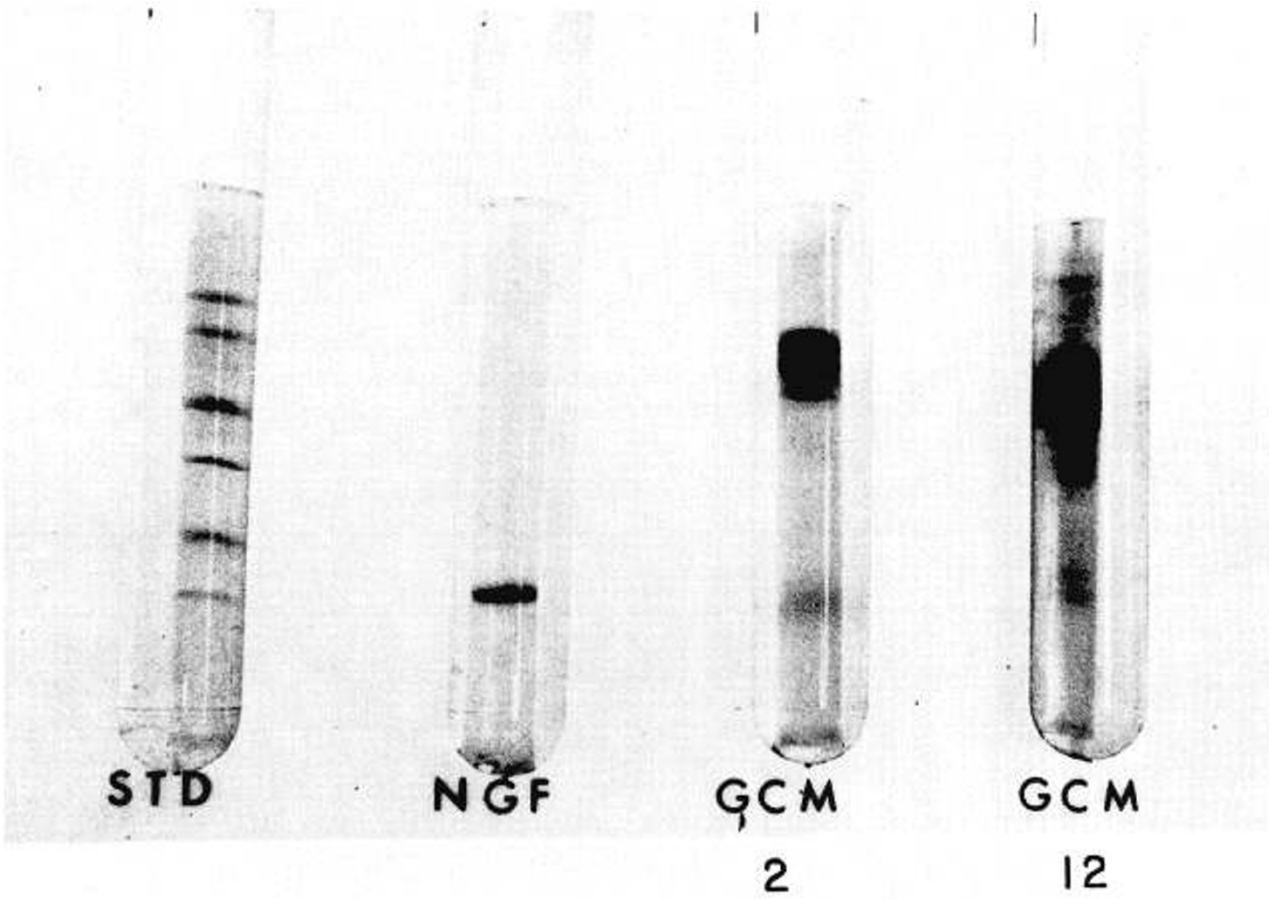

Fig. 3. SDS-PAGE 5–20% linear gradient gels run at 4 mA/gel in Tris-glycine-SDS, pH 8.3. The standard (*STD*) markers from top to bottom were phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin (faint band roughly equivalent to NGF band in tube 2). Tubes 3 and 4 show GCM from 2 day and 12 day cells, respectively.

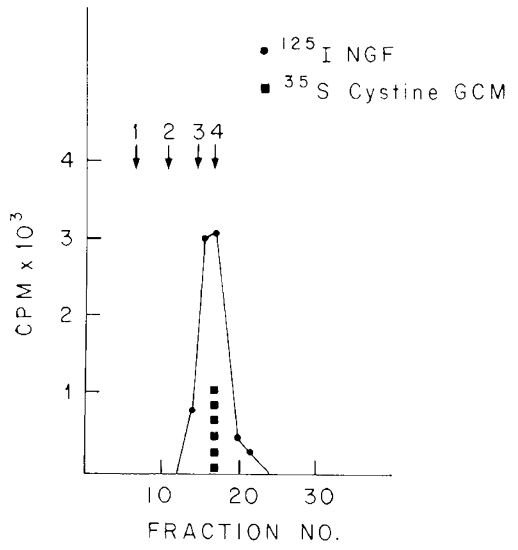

Fig. 4. SDS 15% PAGE gels run with [^{125}I] β-NGF (●) and [^{35}S]-cystine GCM immunoprecipitated with β-NGF antiserum (■) 2-mm slices. Standards represented are *1*, carbonic anhydrase; *2*, trypsin inhibitor; *3*, lysozyme; and *4*, cytochrome C.

Analytical gel filtration of the GCM β-NGF immunoreactivity and [^{125}I] β-NGF gave similar elution patterns, the peaks being one fraction apart (Fig. 5). Both the [^{125}I] NGF and the β-NGF immunoreactivity eluted between the trypsinogen and cytochrome C markers. These peak fractions (29, 30, 31) showed

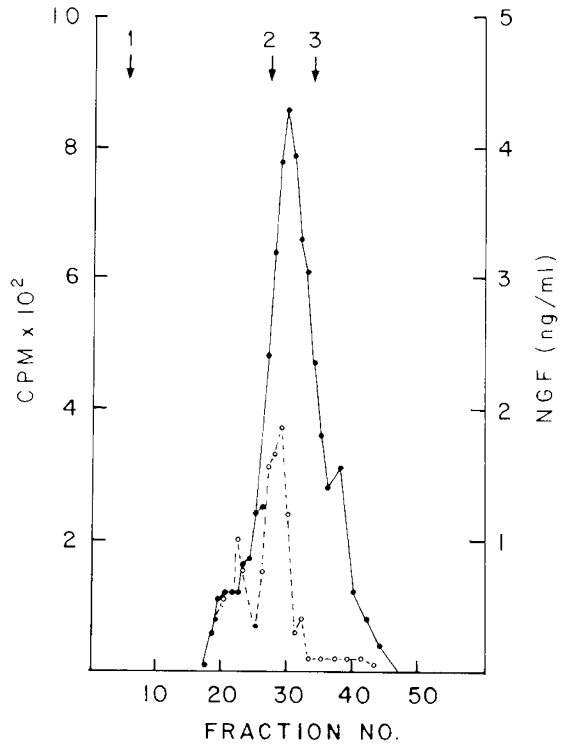

Fig. 5. Sephadex G-100 chromatogram (1 × 50 cm) with PO_4-NaCl-$MgCl_2$ buffer, pH 7.0: ^{125}I β-NGF (○) and GCM (●). Marker standards *1*, blue dextran; *2*, trypsinogen; and *3*, cytochrome C.

approximately 4 ng/ml of NGF-like material by radioimmunoassay. The pooled peak fractions demonstrated bioactivity with the PC-12 bioassay after elution from the column. Analysis of the area under the GCM elution peaks gave a yield of about 45 ng immunoreactive NGF-like material. The shoulder on the iodinated NGF peak is assumed to be aggregated material.

DISCUSSION

The results of this study indicate that newborn mouse astrocytes in culture produce several proteins identifiable in the conditioned culture medium. The SDS-PAGE linear gradient gels show at least seven protein components, five minor and two major, with a molecular weight range (in the reduced form) of approximately 80,000 to 12,000 daltons (Fig. 3). The minor bands in the 2- and 12-day GCM ran similarly to purified mouse submandibular gland β-NGF and α-lactalbumin, suggesting a molecular weight approximating 12,000 daltons and a migration pattern similar to purified β-NGF. This was further supported by the identical mobility of the ^{35}S-cystine labeled GCM and ^{125}I-NGF on the 15% PAGE gels (Fig. 4) and the similar peaks of NGF immunoreactivity in GCM and ^{125}I-NGF on analytical gels (Fig. 5).

Pulse chase labeling studies showed progressive labeling of cellular NGF-LF over 72 h with secretion into GCM between 96 and 120 h (Fig. 2). The PC12 bioassay of GCM in the 50 and 100 μl volumes demonstrated neurite outgrowth which was inhibited by NGF antiserum. The 200-μl antiserum dose did not inhibit bioactivity in the 200 μl GCM samples (Fig. 1), and larger doses were not used. Nonetheless these results clearly indicate NGF-like bioactivity in GCM of both 2- and 12-day astrocytes in culture.

We conclude from these results that astrocytes in developing mouse brain produce a peptide with biochemical, biological, and immunological characteristics similar to mouse submaxillary gland β-NGF. Such results have not been published previously. In the SMG, NGF is produced as a large 7S complex which is dissociated to subunits at a concentration less than 1 μg/ml (35). Whether the NGF-like material in our culture system is produced as a 7S complex is not clear; we have not further characterized the cellular or conditioned material. If the astrocyte NGF is produced as a 7S complex, it may be rapidly dissociated to the active β-NGF subunit in the culture medium.

Norrgren *et al.* (21) previously addressed the issue of whether NGF is secreted by brain cells by studying a normal human astrocyte-like glial cell line. These investigators detected release from the cell pellet and GCM of a factor similar in biological activity and immunological properties to mouse NGF. Although suggestive of production of an NGF-like factor from human glial cells *in vitro*, these results were inconclusive since GCM was not subjected to radioimmunoassay and chromatography analyses were not conducted.

The significance of NGF in central nervous tissue is not clear. A series of recent *in vivo* studies have suggested that endogenous CNS NGF and its receptor may be important for the function of cholinergic neurons. Developing cholinergic neurons of both the basal forebrain and striatum respond to NGF injections (36). Although neurons from these two areas are anatomically and physiologically dissimilar, the fact that both neuron types respond to NGF is of interest. Since NGF does not cross the blood brain barrier, these recent findings further support the likelihood that NGF is produced within CNS tissue. The present results indicating *in vitro* synthesis and secretion of an NGF-like factor in CNS astroglial cells are supportive of the potential role of NGF in the CNS. Our results also provide direct evidence that an NGF-like factor is produced by neonatal brain astrocytes so that a role in brain development is possible.

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