SECRETION OF NERVE GROWTH FACTOR BY CENTRAL
NERVOUS SYSTEM GLIOMA CELLS IN CULTURE

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Recent studies in this laboratory have revealed that a variety of nonneural cells (both transformed and untransformed) synthesize and secrete a protein which is biologically and immunologically similar to mouse submandibular gland nerve growth factor (NGF) (11, 12, 14, 15). We have also presented evidence that mouse neuroblastoma cells (a peripheral nerve tumor) secrete NGF in culture (11) – an observation which establishes that cells of neural origin can synthesize the protein. Since certain neurons of the central nervous system have been shown by biological criteria to respond to NGF (2, 8), it is possible that nonneuronal cells in the central nervous system may secrete NGF for the purpose of exerting a biological influence upon neurons.

Several lines of evidence indicate that glial cells in vitro can stimulate neurite outgrowth by neuronal cells (4, 5, 9) although there is a controversy as to whether these effects stem from the action of NGF. Moreover, it has not been established whether glial cells do in fact produce the protein. For example, Longo and Penhoet have reported that NGF is present in glioma tumors in vivo (7), although Levi-Montalcini has pointed out (6) that these results could have arisen from the presence of fibroblasts within the tumor mass since fibroblasts have been shown to secrete NGF in culture (14). Also, Monard et al. have described a factor produced by glioma cells which promotes process formation by neuroblastoma cells but, unlike NGF, does not stimulate neurite growth by spinal ganglia (10).

To determine whether cells of glial origin produce NGF, we have used a radioimmunoassay, a bacteriophage immunoassay, and biological assays to measure NGF in media conditioned by rat C-6 glioma cells. By all three criteria, the cells secrete a factor which is indistinguishable from mouse submandibular gland NGF. A preliminary account of some of these experiments has been presented (1).

MATERIALS AND METHODS

Cell Cultures

The C-6 glioma line was obtained from the American Type Culture Collection, Rockville, Md. The cells were utilized between passages 37 and 41 and were karyotyped at the beginning and end of the study (courtesy of Dr. Leonard Atkins, Massachusetts General Hospital, Boston, Mass.). Cells were grown (37°C, 5% CO_2) to confluence in 150 cm^2 tissue culture flasks (Corning Glass Works, Science Products Div., Corning, N. Y.) containing 20 ml of Eagle's minimal essential medium (MEM, Flow Laboratories Inc., Rockville, Md.) supplemented with 10% fetal calf serum (Grand Island Biological Company, Grand Island, N. Y.), nonessential amino acids (Flow), gentamycin (50 μg/ml, Schering Corp., Port Reading, N. J.) and Fungizone (3 μg/ml, E. R. Squibb & Sons, Princeton, N. J.). At confluence the medium was removed, and the cells were washed three times with 10 ml of medium without serum and then fed with 20 ml of serum-free medium. After 2-4 days, this medium was removed, dialyzed thoroughly against 0.01 M ammonium acetate, and lyophilized. For biological assays, the dry culture medium was redissolved in MEM and dialyzed against this solvent. For immunological

assays, it was dissolved in and dialyzed against 0.1 M potassium phosphate, pH 7.0.

Preparation of NGF and Antisera

NGF was isolated and purified from male mouse submandibular glands by modifications (12) of the method of Bocchini and Angeletti (3). All preparations were shown to be electrophoretically homogeneous in three solvent systems (12). Antisera to NGF were prepared in rabbits as previously described (12).

Sensory Ganglion Assays

Biological activity was assayed with nine-day chick embryo dorsal root ganglia (11).

Immunoassays

The procedures used for covalently linking NGF to bacteriophage T_4 and for the phage assay have been presented earlier (12). The principle of this assay is that NGF antiserum renders phage-NGF particles noninfective for *Escherichia coli*. Free NGF competes for antiserum in the reaction and can thus be measured quantitatively. The method of preparation of ^{125}I-NGF and the radioimmunoassay procedure used have been published (11). The limit of sensitivity of the phage assay is 1 ng/ml and that of the radioimmunoassay is 0.5 ng/ml.

Normal rabbit γ-immunoglobulin (IgG) and IgG from sera of rabbits immunized with NGF were isolated by the following procedure. Sera were diluted with an equal volume of 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, and the resulting solution was brought to 50% saturation with $(NH_4)_2SO_4$. The precipitate, collected by centrifugation, was redissolved in and dialyzed against 0.01 M sodium phosphate 0.015 M NaCl, pH 7.5, and applied to a column of DE-52 (Whatman Inc., Clifton, N. J.) equilibrated with the same solvent. The unabsorbed fraction was collected and stored frozen. Anti-NGF IgG prepared in this way strongly inhibited the action of NGF upon neurite outgrowth from spinal ganglia, whereas nonimmune IgG had no inhibitory effect.

Protein concentrations were measured both by ultraviolet spectrophotometry (λ = 280 nm) and by the Lowry procedure.

RESULTS AND DISCUSSION

Table I presents results of bacteriophage assays of serum-free concentrated glioma culture fluids as a function of time in culture. After 24 h, an immunoreactive substance (based upon mouse-NGF standards) has been secreted into the medium. That this material did not arise from residual serum is shown by the fact that immunoreactivity is not detected after only 2-3 h in culture and by the fact that we have been unable to detect the presence of NGF in fetal calf serum either immunologically or biologically (11).

To compare results obtained by the phage assay and by radioimmunoassay, we grew glioma cells for 4 days in serum-free medium which was concentrated as described above. Table II reveals that both assays yield virtually identical values.

Figure 1a reveals that concentrated glioma culture fluids also contain a factor which induces extensive neurite outgrowth from sensory ganglia. By radioimmunoassay, this concentrated culture supernatant solution contained 4 ng/ml of immunoreactive material, arising from 200 ml of culture fluid. Figure 1b illustrates that the biological response is unaffected by the presence of 14.5 μg/ml of normal rabbit IgG, whereas Figure 1c reveals that 14.5 μg/ml of anti-NGF IgG markedly inhibits (but does not abolish) neurite extension. The finding that a few neurites grow in the presence of antibody may indicate that other growth

Table I

Immunoassays of C-6 Glioma Culture Media as a Function of Time in Culture

Time	NGF
(h)	(ng/ml)*
2	Not detected
3	Not detected
24	0.09 ± 0.01
49	0.065 ± 0.005
72	0.065 ± 0.004

* Cultures were grown in serum-free media (20 ml) for the times indicated, and media were concentrated as described in the text. Values shown represent nanogram equivalents (± SEM) of immunoreactive material per milliliter of original unconcentrated medium, based upon the phage immunoassay, with mouse submandibular gland NGF as standards. Quadruplicate assays at each time period were performed with solutions which had been concentrated 100-fold.

Table II

Analysis of a C-6 Glioma Culture Medium by Radioimmunoassay and Phage Immunoassay

Method	NGF
	ng/ml*
Radioimmunoassay	0.05 ± 0.007
Phage assay	0.07 ± 0.009

* Values represent NGF-equivalents in nanograms per original milliliter of culture fluid based upon mouse NGF standards. Assays were performed in triplicate, and the standard errors of the mean are given. Confluent cultures were maintained for 48 h in serum-free MEM. This culture fluid was concentrated 40-fold for assay purposes as described in the text.
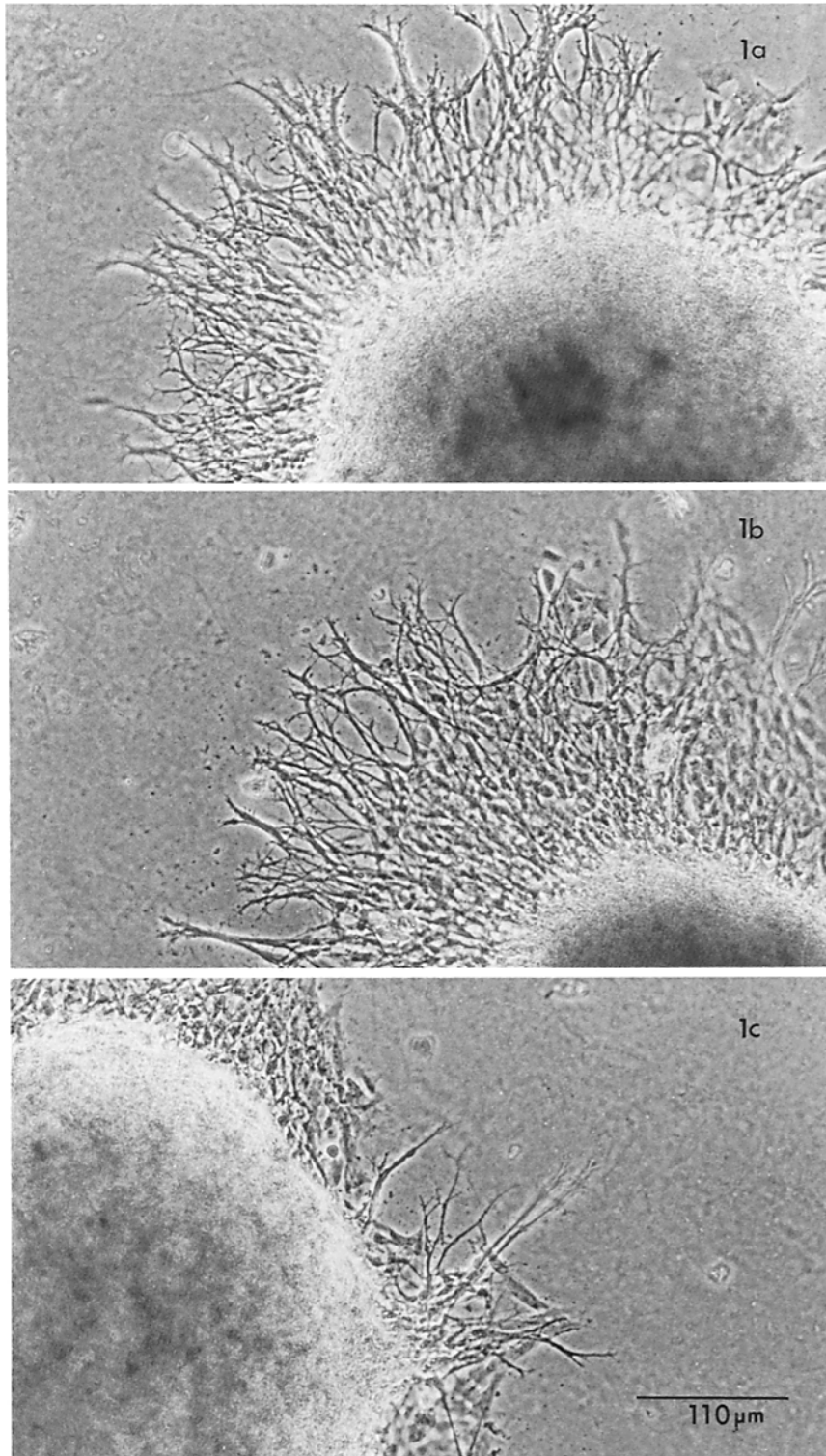
FIGURE 1   Effects of serum-free glioma culture fluid upon chick embryo dorsal root ganglia. Sensory ganglia from nine-day embryos were explanted onto collagen-coated cover slips and treated with MEM containing 10% fetal calf serum and: (a) concentrated glioma culture fluid containing 4 ng/ml NGF-immunoreactive equivalents by radioimmunoassay; (b) same as (a) plus 14.5 μg/ml normal IgG; (c) same as (a) plus 14.5 μg/ml IgG prepared from rabbit antisera to NGF. Ganglia were examined after 19 h of incubation in a humidified atmosphere containing 5% CO_2 at 37°C. Phase contrast micrographs, × 180.

factors are present in the medium (4, 5, 9). (The results presented in this figure are representative of and were obtained from a study in which ten ganglia were treated with normal rabbit IgG and ten ganglia were treated with anti-NGF IgG. In addition, 34 ganglia were treated with C-6 conditioned medium without IgG, and all of them showed responses similar to that depicted in Figure 1a.)

The following studies establish that the appearance of NGF in culture media arises from net synthesis of the protein by C-6 cells. Two sets of identically incubated cultures were grown to confluence. Medium was removed and both sets were washed and treated with serum-free medium as described in Materials and Methods. From one set of cultures (zero time set) this culture medium was immediately removed, dialyzed exhaustively against 0.01 M ammonium acetate, and lyophilized. Cells were removed from the culture dishes in the presence of 0.003 M EDTA, pH 7.0, collected by centrifugation, and frozen. The second set of cultures was maintained for an additional 72 hours in serum-free media, after which time cells and media were treated as described above. The lyophilized culture fluids were redissolved in 0.1 M potassium phosphate, pH 7.0, and dialyzed against this solvent. To measure intracellular NGF, we mechanically disrupted the cells (0.1 M potassium phosphate, pH 7.0, ground-glass homogenizer), and the resulting cell suspensions were clarified by centrifugation and dialyzed against the phosphate buffer. Both cell culture media and intracellular NGF were then measured by radioimmunoassay. Fig. 2 reveals that, as expected, no NGF was detected extracellularly at zero time. Over the subsequent 72 h, intracellular NGF remained constant while extracellular levels increased significantly. These results could have arisen only from net synthesis and secretion of the protein in the culture system.

Earlier studies of Burnham et al. (4) have shown that when dissociated sensory ganglionic neurons are cultured together with a sufficiently high density of nonneuronal cells, then both neuronal fiber production and neuronal survival are enhanced. While these effects can be inhibited by antibody to mouse NGF (13), it is not clear which cells in culture produced the NGF-like factor. The factor could have arisen from glia, from fibroblasts in the nonneuronal population (14), or from the neurons themselves. The results of the present
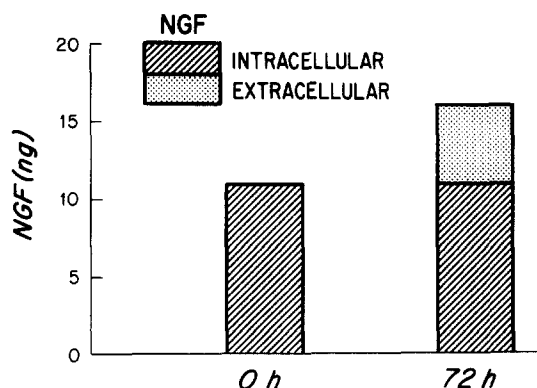

FIGURE 2 Net synthesis of NGF by glioma cells. Two sets of cultures were used to measure the intracellular and extracellular levels of NGF by radioimmunoassay immediately (zero time) after treating cells with fresh serum-free media and after 72 h in culture. Cells and media were prepared as described in the text. After 72 h, total NGF in the system has increased.

study do not establish whether a normal population of glia cells secrete NGF, but they do reveal that a glial tumor cell line secretes a macromolecule which is closely similar biologically and immunologically to mouse submandibular gland NGF.

It should be noted that the C-6 glioma line was derived originally from an N-nitrosomethylurea-induced tumor of the central nervous system. Since NGF has been shown to affect central neurons in two different systems (2, 8), it could be that one function of central glial cells is to produce nerve growth factor in the brain.

SUMMARY

Bacteriophage immunoassays, radioimmunoassays, and biological assays have been used to measure levels of NGF in media conditioned by rat C-6 glioma cells in culture. By all three criteria, these cells secrete a macromolecule which is indistinguishable from mouse submandibular gland NGF.

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