Secretion of a Nerve Growth Factor by Mouse Neuroblastoma Cells in Culture 
(conditioned culture medium/fibroblasts)

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ABSTRACT Analyses of supernatant solutions from mouse C1300 neuroblastoma cultures by two independent immunoassays reveal that these cells secrete a factor which is immunochemically similar to mouse submaxillary gland nerve growth factor. The neuroblastoma factor is also biologically active in inducing neurite outgrowth from embryonic sensory ganglia—an effect that is completely blocked by specific antibody to nerve growth factor. Neuroblastoma cells are known to be functionally responsive to nerve growth factor, and the observation that they secrete a molecule like it may mean that these cells require or utilize the factor during growth in culture.

Recent studies in this laboratory have shown that several lines of cells in culture secrete a biologically active nerve growth factor which is immunologically similar to mouse submaxillary gland nerve growth factor (NGF). These lines include mouse L cells, 3T3 cells, SV3T3 cells (3T3 cells transformed by simian virus 40) (1), and primary chick embryo fibroblasts (2). Moreover, chemical studies have shown that mouse L cells secrete a factor that possesses the chromatographic and electrophoretic properties of NGF (3). Taken together, these observations led us to suggest that secretion of NGF may be a general property of fibroblasts; that NGF may play a role in the biological function of granulation tissue; and that it may be responsible for one or more of the cell-growth-promoting effects of conditioned tissue culture medium (1, 2).

In addition to the well-known biological effects of NGF upon sympathetic and sensory ganglia (4), several lines of evidence indicate that this growth factor also acts upon neuroblastoma cells in culture. For example, NGF stimulates the synthesis of acetylcholinesterase in mouse neuroblastoma (5), as well as the growth of nerve processes and synthesis of microtubule protein by human neuroblastoma cells (6). Further, mouse neuroblastoma cells have been shown to bind NGF upon their membrane surfaces (7).

These findings are pertinent to recent studies on the effects of glial cells upon neuronal cells in culture. Glial cells have been shown to promote nerve fiber production by dissociated chick dorsal root ganglion cells (8), as well as to induce morphologic differentiation of mouse neuroblastoma cells (9). The fact that both human glioblastoma (10) and rat (11) glioma cells secrete a nerve growth factor immunologically similar to NGF could be the basis for these observations.

Although secretion of NGF may be a general property of fibroblasts, at least in vitro, the biologic purpose(s) for its secretion are unknown. On the other hand, in light of (1), the close association of glial and neuronal cells in vivo; and (2), the secretion of a molecule similar to NGF by glial cells, it could be that one function of glial cells is to supply neurons with NGF. This line of reasoning led us to predict that a neuronal cell line in culture would not secrete NGF. Accordingly, we have studied neuroblastoma cells in culture and we find to our surprise that these cells, which are biologically responsive to NGF, also secrete a biologically active nerve growth factor which is immunochemically similar to NGF.

MATERIALS AND METHODS

Preparation of NGF. NGF was prepared from male mouse submaxillary glands by modifications (1) of the method of Bocchini and Angeletti (12). Preparations were examined by gel electrophoresis in three solvent systems and by immunoelectrophoresis and were shown to be homogeneous, as described by Oger et al. (1).

Bioassay of NGF. Chick embryo (8-days-gestation) dorsal root ganglia were used to evaluate the neurite outgrowth-producing effect of NGF. Ganglia were placed upon collagen-coated coverslips in petri dishes; the nutrient culture medium contained 90% Eagle’s minimal essential medium with Earle’s balanced salt solution (MEM, Flow Labs, Rockville, Md.) supplemented with nonessential amino acids (Flow) and either 10% heat-inactivated calf serum (Gibco) or rabbit serum. Preparations were examined microscopically after 15-hr incubation (humidified atmosphere containing 5% CO₂ at 37°C).

Bacteriophage Immunoassay of NGF. NGF was covalently linked to a wild-type bacteriophage strain of (T₄Dr⁺), and procedures for purification of the bacteriophage T₄-NGF conjugates, as well as the immunoassay, have been presented (1). In this assay, antibody directed against NGF inactivates the phage-NGF conjugate, rendering it noninfective for Escherichia coli. Free NGF competes for antibody in the reaction, and this forms the basis for the assay, which can detect as little as 2.5 ng/ml of NGF.

Rabbit antiserum to purified NGF were prepared as described (1).

Radioimmunoassay of NGF. ¹²⁵I-NGF was prepared by modifications of the method of Greenwood et al. (13). To 4 μg of NGF dissolved in 20 μl of 1 M potassium phosphate, pH 7.0, was added 50 μl of carrier-free Na¹²⁵I containing 0.5 mCi

Abbreviations: NGF, male mouse submaxillary gland nerve growth factor; MEM, minimal essential medium with Earle’s balanced salt solution.
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TABLE 1. Bacteriophage and radioimmunoassays of concentrated neuroblastoma culture solutions

<table>
<thead>
<tr>
<th>Method</th>
<th>NGF (ng/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioimmunoassay</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>Bacteriophage</td>
<td>0.18 ± 0.03</td>
</tr>
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Cells were grown for 4 days in serum-free medium and the supernatant solution (780 ml) was concentrated as described in the text.

* Values represent nanograms of immunoreactive material per ml of original unconcentrated culture fluid, (±SEM) based upon assays of NGF standards, in triplicate.

(New England Nuclear Corp.). Chloramine-T (Eastman), 10 μl of a 2.3 mg/ml solution in H2O, was used to oxidize 125I-. Reaction was stopped after 30 sec by addition of 100 μl of Na2S2O4 (0.6 mg/ml in H2O). 125I-NGF was separated from other reaction products by gel filtration (Sephadex G-25) in a solvent containing 0.1 M potassium phosphate, pH 7.0, and 1 mg/ml of five times crystallized bovine serum albumin (Penx). Specific activities of labeled NGF have ranged between 0.1 and 0.5 g-atom I per mole of NGF.

Solutions for radioimmunoassay contained 100 μl of a 4 ng/ml solution of 125I-NGF, 100 μl of standard solutions of NGF (5–250 ng/ml), 100 μl of a 1:1000 dilution of NGF antiserum, 10 μg of rabbit IgG—in a total volume of 0.9 ml of 0.1 M potassium phosphate, pH 7.0, containing 1.0 mg/ml of bovine serum albumin. These solutions were incubated for 18 hr at 4°C, at which time 100 μl of a suitable dilution of goat antibody against rabbit IgG was added. After incubation for 4 hr at 4°C, samples were centrifuged for 30 min at 1500 × g, and 200 μl of the supernatant solution was removed for measurement of radioactivity (Packard gamma spectrometer). The limit of sensitivity of this assay is 1.0 ng of NGF/ml.

Culture of Neuroblastoma Cells. Mouse neuroblastoma cells, line C-1300 2a, were obtained from the American Type Tissue Culture Collection, Rockville, Md. Cells were grown to confluence in 75 cm² Falcon tissue culture flasks in the presence of MEM plus 50 μg/ml of gentamycin, supplemented with 10% heat-inactivated fetal calf serum in an humidified atmosphere of 5% CO2 at 37°C. Cells were fed twice per week. To prepare media for assay, the serum-containing medium was removed, and cells were washed twice with 5 ml of serum-free medium. Cells were then incubated for 4 days in 10 ml of MEM without serum. This medium (total volume 780 ml) was collected, dialyzed thoroughly against 0.01 M ammonium acetate, and lyophilized. The dry residue was redissolved in 1.0 ml of 0.1 M potassium phosphate, pH 7.0, and dialyzed against this solvent for immunoassays. For bioassays, aliquots were dialyzed against MEM.

RESULTS

Table 1 presents results of both radioimmunoassay and bacteriophage immunoassays of concentrated neuroblastoma culture supernatant solutions. After 4 days in culture, the original unconcentrated culture medium contained 0.15 ng/ml (radioimmunoassay) and 0.18 ng/ml (phage assay) of a nondialyzable NGF-immune serum. Neurite extension is totally inhibited. Substitution of 10% heat-inactivated fetal calf serum for normal rabbit serum gave results identical to those shown in the top and middle photographs. Phase contrast photomicrographs, ×172.5.
able macromolecule that reacts with antibody to mouse submaxillary gland NGF.

To examine the biological activity of these solutions, the concentrated culture fluid was dialyzed thoroughly against MEM and tested in the sensory ganglia assay system. Fig. 1 reveals that these solutions induce neurite outgrowth typical of NGF after 15 hr in culture.

Phage and radioimmunoassays of the concentrated cell supernatant solution yield values of 140 ng/ml and 115 ng/ml, respectively, of immunoreactive material. Semiquantitative sensory ganglion assays of serial dilutions of this solution, when compared to assays of serial dilutions of NGF, gave a value of 120 ng/ml. The correspondence between bioassay and both immunoassays suggests that the neuroblastoma factor is closely similar to NGF itself.

To see whether antibody against NGF would block the biologic activity of the neuroblastoma factor, 45 μl aliquots of concentrated culture medium were treated with 5 μl of rabbit NGF antiserum or with nonimmune rabbit serum. Fig. 1 reveals that rabbit antiserum to NGF completely blocks the nerve growth effect, whereas normal rabbit serum does not.

**DISCUSSION**

The observations presented above indicate that neuroblastoma cells in vitro synthesize and secrete a nerve growth factor which is similar (and perhaps identical) to mouse submaxillary gland NGF. An alternative explanation for the results is that the NGF-like factor is derived from fetal calf serum, concentrated by the cells, and then slowly released into the serum-free medium. While this possibility is difficult to exclude rigorously, the following lines of evidence suggest that this mechanism is an unlikely one. (1) We have never detected an immunoreactive substance either by radioimmunoassay or phage immunoassay in the many different lots of fetal calf serum that have been analyzed. (2) The limit of sensitivity of the radioimmunoassay is 1 ng/ml. This means that the cells were in contact with serum (10% by volume) which could have contained no more than 0.1 ng/ml of immunoreactive material. Taking into account the total volume of serum, this is considerably less than the total NGF-like factor liberated by the cells into serum-free medium (Table 1).

In addition to the observation that neuroblastoma cells release a biologically active NGF-like substance, substantial evidence now indicates that these cells also respond to NGF (5-7) and that the primary receptors for the growth factor are associated with the plasma membranes of sensitive cells (14, 15, 16, 17). There are several possible explanations for these two pieces of information. One is that certain cells within the clone secrete an NGF-like molecule to which other cells respond. Alternatively, a given neuroblastoma cell may secrete the growth factor at one stage of the cell cycle and respond to it at another stage. Indeed, evidence from studies with synchronized neuroblastoma cells suggests that binding of 125I-NGF to the cell surface is maximal during the late G1 and early S phases of the cell cycle (7). Moreover, the studies of Cifone and Defendi indicate that a macrophage growth factor is secreted by L cells as a function of the cell cycle. This factor is present intracellularly during all phases, but is transferred to the cell surface during S-phase and released into the medium after metaphase (17). Since L-cells also secrete an NGF-like factor (1), it could be that NGF secretion fluctuates during the cell cycle. Finally, it is conceivable that a given cell may synthesize NGF, secrete it, bind it, and functionally respond to it as part of an autoregulatory mechanism.

In 1964, Burdman and Goldstein reported that sera from children with neuroblastoma contained increased amounts of a nerve growth factor as judged by sensory ganglion assays (18). Subsequent studies have not confirmed this finding (19, 20). The observation that mouse neuroblastoma cells in culture secrete NGF or an NGF-like factor suggests that a study of sera from human neuroblastoma patients with immunologic methods might reveal elevated levels of this factor.

Neuroblastoma may now be added to the list of cell lines, both malignant and nonmalignant, which have been shown to secrete a molecule similar to NGF (1, 2). Whether the capacity to secrete and to utilize NGF is a property not only of neuroblastoma, but of other cells which produce it is not known.

**Note Added in Proof.** The following studies provide further evidence that the neuroblastoma nerve growth factor is synthesized and secreted by the cells and that it is not derived from fetal calf serum.

Three groups of flasks were identically inoculated with neuroblastoma cells. Cells were grown to confluence in MEM supplemented with 10% fetal calf serum, washed with MEM, and fed 10 ml of MEM without serum. The medium from group 1 was immediately collected and concentrated as described in the Materials and Methods section. The cells in this group were removed from the flasks by ten minute treatment with 0.1% Versene, washed with Hanks' balanced salt solution, collected by centrifugation, and frozen. Group 2 cells were incubated in serum-free medium for four days; group 3 cells were incubated for the same time period in medium containing 5 ng/ml of cycloheximide. After incubation, the conditioned medium and the cells of groups 2 and 3 were treated identically to those in group 1. All cell pellets were thawed and disrupted by homogenization in 1 ml of 0.1 M potassium phosphate, pH 7.0. Insoluble material was removed by centrifugation (15 min at 2000 × g) and the supernatant solutions, as well as the concentrated media from each group, were examined by radioimmunoassay to determine the amount of NGF present. By these methods, 70 ng of NGF was found to be associated with the cell pellet at the initiation of the test period (group 1) and no NGF was detected in the culture medium. The cells in group 2 contained 63 ng of NGF while the medium contained 113 ng. This represents a marked increase in the amount of NGF present in the total system (cells plus medium) after four days' incubation in serum-free medium. Cells treated with cycloheximide (group 3) contained 19 ng of NGF while the medium contained only 12 ng.

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