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# Synthesis of Nerve Growth Factor by L and 3T3 Cells in Culture

**(conditioned culture medium/bacteriophage immunoassay/fibroblasts)**

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**ABSTRACT** **Mouse submaxillary gland nerve growth factor (NGF) has been covalently joined to bacteriophage and the resulting phage conjugates remain biologically active in stimulating neurite extension from sensory ganglia. A sensitive bacteriophage immunoassay has been developed to measure concentrations of NGF as low as 1 ng/ml. With this method, we find that mouse L and 3T3 cells in culture produce a biologically active nerve growth factor that is immunologically similar if not identical to mouse submaxillary gland NGF. Since L cells are known to be a source of "conditioned medium" for tissue culture, it could be that one or more of the conditioning factor activities secreted by these cells are due to NGF itself.**

In the study presented below, several lines of evidence indicate that mouse L and 3T3 cells in culture produce a biologically active nerve growth factor that is similar to (and possibly identical with) the nerve growth factor (NGF) isolated from male mouse submaxillary glands.

The L cell fibroblast strain was derived in 1943 from explanted mouse C3H subcutaneous and adipose tissue which had been treated *in vitro* for several months with 20-methylcholanthrene (1). In 1948, L-cells were cloned by Sanford *et al.* and a permanent line (no. 929) established (2). Several sublines of clone 929 have been prepared; one of them (the $L_2$ subline) was used in the present study. (See ref. 3 for a detailed description of the $L_2$ line.)

L-cells injected into newborn C3H mice produce malignant sarcomas, and in this connection, it will be recalled that the first description of nerve growth factor activity arose from studies with two mouse sarcomas (sarcomas 180 and 37) (4–6). Moreover, extracts of experimentally induced mouse granuloma tissue also display a nerve growth factor activity *in vitro* (7).

These studies are pertinent to the question of where NGF is synthesized in the mouse. For example, the male mouse submaxillary gland contains large amounts of NGF (8) and the growth factor may be synthesized there (9, 10). On the other hand, the radioimmunoassay studies of Hendry and Iverson demonstrate that submaxillary gland cannot be the sole source of NGF in the adult mouse, since serum levels of the growth factor return to normal 2 months after removal of both submaxillary glands (11). Other sources of NGF have not been identified in the normal mouse.

Abbreviations: NGF, nerve growth factor; SDS, sodium dodecyl sulfate; MEM, minimal essential medium with Earle's balanced salt solution; $T_4$–NGF, conjugate of NGF covalently linked to $T_4$ bacteriophage; SV 3T3 cells, simian-virus-40-transformed 3T3 mouse fibroblasts.

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## MATERIALS AND METHODS

*Preparation of Pure NGF.* NGF was purified from a homogenate of 800 male mouse submaxillary glands by two modifications of the method described by Bocchini and Angeletti (12). (*i*) After addition of streptomycin to the clarified gland homogenate (12), saturated aqueous $(NH_4)_2SO_4$ (Schwartz/Mann) was added and the fraction precipitating between 52 and 71% (4°) saturation collected by centrifugation. (*ii*) The other modification involves the carboxymethyl cellulose chromatography step (12). The NGF preparation, dissolved in 0.05 M sodium acetate, pH 5.0, was applied to a 2.4 × 5.4-cm column of CM-52 (Whatman) equilibrated with the same buffer. A step gradient to 0.15 M NaCl was applied to elute most contaminating proteins. Next, a linear NaCl gradient (0.15–2.0 M NaCl; total volume 600 ml; NaCl dissolved in 0.05 M sodium acetate, pH 5.0) was used to elute NGF, which emerged as a single peak at 0.5 N NaCl. Fractions were pooled and protein concentration was measured by the Lowry procedure. Purified NGF was stored in 5-ml aliquots at −15°. These preparations of NGF displayed high biological activity when assayed using sensory ganglia.

*Gel Electrophoresis.* Polyacrylamide gel electrophoresis of the NGF subunit was carried out with the sodium dodecyl sulfate (SDS)–phosphate system of Weber and Osborn (13), the SDS–borate system of Neville (14), and with the high resolution acetic-acid system described by Panyim and Chalkley (15). Gels were stained with Coomassie Brilliant Blue and destained with 40% methanol: 10% acetic acid.

*Bioassay of NGF.* Chick embryo (10–14 days) dorsal root ganglia were used to estimate the neurite outgrowth-producing effect of NGF as previously described (16). 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, Gibco) and 10% heat-inactivated (56°, 30 min) fetal-calf serum (Gibco). Preparations were examined microscopically after 18- to 24-hr incubation in an humidified atmosphere containing 5% $CO_2$ at 37°.

*Cell Cultures.* The subline $L_2$ (from clone 929) was kindly provided by Dr. L. S. Sturman in December 1972 and has since been maintained by serial subculture. Feeding medium (90% MEM, 10% fetal-calf serum) was changed weekly. Cells were grown in 75-cm² plastic Falcon tissue culture flasks. Under these conditions, doubling time is approximately 17 hr. Cells were removed from the flask surface with 0.25% trypsin solution (Gibco).

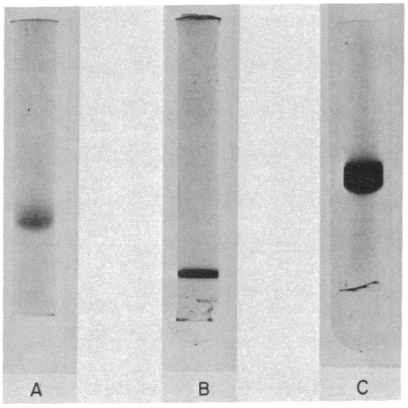


FIG. 1. Gel electrophoresis of purified NGF. Migration is from top to bottom; (*A*): 26 μg of NGF, SDS–phosphate system (14); (*B*): 15 μg of NGF, SDS–borate system (13); (*C*): 210 μg of NGF, acetic-acid system (15).

Mouse Balb/c 3T3 cells, clone A31, and simian-virus-40-transformed A31 cells (SV 3T3) were kindly provided by Dr. Paul Black. The origin and history of these cells is described elsewhere (17). They were maintained by serial subculture in the presence of 90% McCoy's medium (Gibco),/10% fetal-calf serum.

*Bacteriophage Immunoassay of NGF.* The immunoassay for NGF is based upon studies originally presented by Mäkelä (18). The basic feature of this assay is that when bacteriophage is covalently coupled to an antigenic hapten, antibody directed against the hapten will render the phage-hapten conjugate noninfective for its host bacterium.

A pure wild-type strain ($T_4Dr^+$, a gift from Dr. Jonathan King) was used to prepare (19) a $T_4$ stock containing $3.3 \times 10^{12}$ plaque-forming units (PFU) per ml of solvent. The host bacterium used both for stock preparation and for phage assays was a strain (HMS-9) of *Escherichia coli* B (courtesy of Dr. Charles Richardson). $T_4$ was purified by differential centrifugation as described by Adams (19). All preparations displayed a single sedimenting component when examined by sucrose density gradient centrifugation (28 ml of a 5–20% linear sucrose gradient; 41,000 × *g* for 40 min at 5°).

Glutaraldehyde was used to couple NGF to $T_4$ by modifications of the procedure of Fuchs *et al.* (20). All conjugates used in the present study were prepared as follows. To 50 μl of $T_4$ ($3.3 \times 10^{12}$ PFU/ml) was added 50 μl containing 0.5 mg of NGF dissolved in 0.05 M sodium acetate, 0.5 M NaCl, pH 5.0. This mixture was treated with 25 μl of freshly *in vacuo* redistilled glutaraldehyde (Fisher) 0.05% (w/w) in water for 15 min at 24°. To stop the reaction, 1 ml of M-21 [Bactopeptone (Difco), 5 g; glucose, 1 g; NaCl, 5 g; Nutrient Broth (Difco), 8 g; per liter of $H_2O$, pH 7] was added. The $T_4$-NGF conjugate was collected by centrifugation at 33,000 × *g* for 60 min, washed three times with M-9 ($NH_4Cl$, 1.0 g; $MgSO_4$, 0.13 g; $KH_2PO_4$, 3.0 g; $Na_2HPO_4$, 6 g, per liter of $H_2O$, pH 6.8), resuspended in 1 ml of M-9, and recentrifuged at 33,000 × *g* for 60 min. The pellet was again resuspended in 1 ml of M-9 and recentrifuged. The final pellet was resuspended in 0.6 ml of M-9 and stored at 4°. Absorbance (λ = 260 nm) together with plaque-forming measurements (19) revealed that 76% of the phage particles were rendered noninfective by the coupling reaction. The final infective phage concentration of this stock $T_4$-NGF conjugate was $1.52 \times 10^{10}$ PFU/ml.

*Preparation of Antisera.* Rabbits were immunized by foot pad injection of 0.2 mg of NGF in 0.4 ml of complete Freund's adjuvant. An intravenous booster injection (0.1 mg) was given 7 weeks later, and the animals were bled the following week. Normal rabbit IgG was purified by DEAE-cellulose chromatography (21) and was used to prepare an antiserum in the goat. A goat was injected intramuscularly with 3 mg of rabbit IgG in complete Freund's adjuvant. Booster injections (3 mg of IgG intramuscular) were given after 8, 12, and 16 weeks, and the animal was bled at 17 weeks. This antiserum yielded a single precipitin arc upon immunoelectrophoresis with normal rabbit serum.

*Immunoelectrophoresis.* Macroimmunoelectrophoresis was performed with 1% Difco agar-noble (sodium barbital buffer, 0.05 M, pH 8.2; 5 V/cm for 4 hr) (22).

## RESULTS

Fig. 1*A* and *B* presents SDS-gel electrophoretic profiles of the NGF used in this study. Only a single electrophoretic component was detected. Molecular weight markers (bovine-serum albumin, Pentex; cytochrome *c*, Sigma) were used to measure the molecular weight of the bands shown in Fig. 1. A value of 15,000 g/mol was obtained. The mass of the NGF subunit from sequence studies is 13,259 g/mol (23). Fig. 1*C* illustrates a deliberately overloaded gel of NGF (210 μg) in the acetic-acid system (15).

Immunoelectrophoretic studies also revealed that the NGF preparation is homogeneous; when anti-NGF antiserum was reacted both with NGF and with a crude homogenate of male mouse submaxillary gland, only a single precipitin band was detected.

*Biological Activity of $T_4$–NGF.* $T_4$-phage conjugates exhibited neurite outgrowth-promoting effects when tested in the sensory ganglia assay system. This property did not arise from contaminating free NGF, since repurification of $T_4$–NGF revealed that all of the biological activity sedimented with the phage particles at a rate of approximately 1000 S. Phage without coupled NGF did not promote neurite outgrowth. These results are consistent with, but do not prove, the hypothesis that NGF may act upon the neuronal membrane (see also refs. 24 and 25).

*Phage-immunoassay of NGF.* Fig. 2 presents a plot of plaque-forming units versus log dilution of anti-NGF antiserum. For this experiment, solutions of $T_4$–NGF were treated first with anti-NGF antiserum and then with a goat antiserum directed against rabbit gamma globulin as described in the *legend* to Fig. 2. As shown in Fig. 2, high concentrations of anti-NGF antisera render 85% of the potentially infective phage particles noninfective. As the antibody concentration is lowered, more phage become infective. Using this method, we have detected anti-NGF activity with serum dilutions as high as $1:10^5$. Nonimmunized rabbit sera exhibit no effect upon $T_4$–NGF infectivity under conditions identical with those given for Fig. 2.

The immunoassay for NGF depends upon competition between free NGF and the $T_4$–NGF conjugate for antibody to NGF. In initial studies, several systems were examined to maximize the sensitivity of the reaction. Best results were ob-

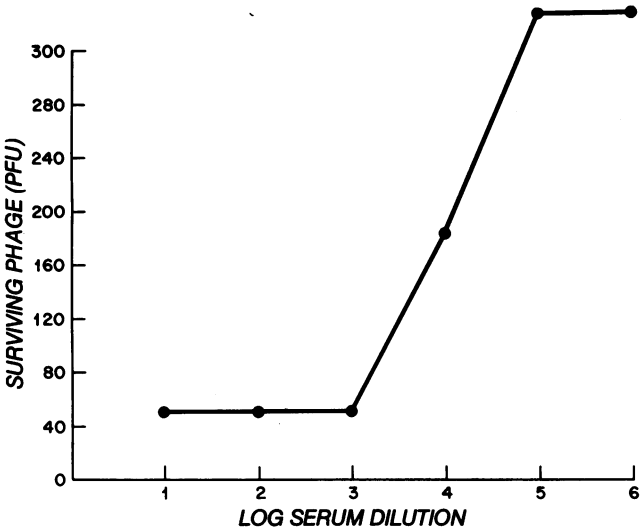


FIG. 2. Inactivation of $T_4$–NGF by anti-NGF serum. Each sample contained 0.1 ml of $T_4$–NGF ($9.6 \times 10^3$ PFU/ml) and 0.1 ml of serial dilutions (in M-21) of NGF antiserum. After 4 hr at 25°, 0.1 ml of a 1:10 dilution (in M-21) of goat anti-rabbit serum was added. Samples (0.1-ml) were plated for assay 20 min later.

tained under the following conditions. To several 0.1 ml samples of $T_4$–NGF at a concentration of $1.5 \times 10^4$ PFU/ml were added 0.1-ml aliquots of solutions containing various concentrations of NGF, and 0.1 ml of a 1:1000 dilution of anti-NGF antiserum. (Both NGF and antisera solutions were diluted with M-21.) After incubation for 16 hr at 4°, 0.1 ml of a 1:10 dilution (in M-21) of goat anti-rabbit antiserum was added to each sample. These mixtures were incubated at 4° for an additional 6 hr, and 0.1-ml aliquots were withdrawn for phage assay by the plating method (19).

The data presented in Fig. 3 demonstrate that free NGF competes with $T_4$–NGF for anti-NGF. At high concentration, free NGF totally protects $T_4$–NGF phage particles from inactivation. With this method, we can detect as little as 1 ng/-ml of NGF.

*Production of NGF by $L_2$ Cells.* L Cells, as well as other cells in culture, are known to produce biologically active factors

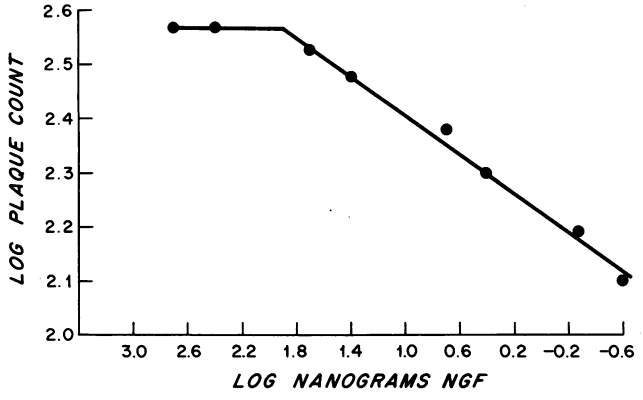


FIG. 3. Phage immunoassay of NGF. Each sample contained 0.1 ml of $T_4$–NGF ($1.5 \times 10^4$ PFU/ml), 0.1 ml of an NGF solution, 0.1 ml of a $10^3$ dilution of anti-NGF serum. Samples were incubated at 4° for 16 hr. A 1:10 dilution of goat anti-rabbit serum was added, solutions were incubated 6 hr at 4° and then 0.1-ml samples were plated.

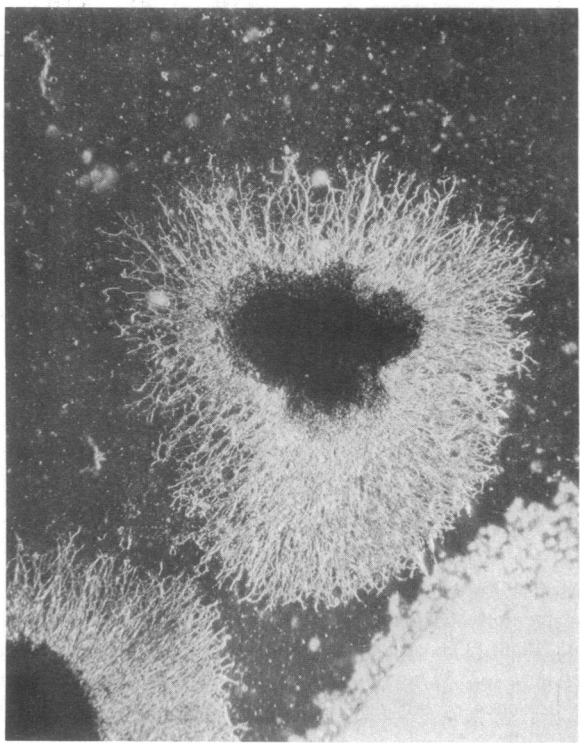

FIG. 4. $L_2$ cell stimulation of ganglionic neurite extension. Neurite outgrowth can be seen to be directed predominantly toward the explanted $L_2$ cells, which lie within the *right* lower corner of the photomicrograph, taken after 18 hr of culture. ×500.

which can influence cell proliferation [see, e.g., Shodell (26)]. To see whether $L_2$ cells might promote neurite outgrowth from explanted sensory ganglia, cultures containing both $L_2$ cells and ganglia were prepared. For this purpose, three to six chick-embryo (10–14 days) dorsal root ganglia were placed on a collagen-coated coverslip together with about $10^6$ $L_2$ cells which had been washed with MEM. After 18–24 hr in culture, we observed that not only did the $L_2$ cells stimulate ganglionic neurite extension, but also that the outgrowth was asymmetrically distributed. That is, the most dense outgrowth occurred predominantly from those regions of ganglia which were spatially nearest to the $L_2$ cells. Fig. 4 illustrates this behavior, which is similar to that observed when ganglia are treated asymmetrically with NGF (27). These results indicate that mouse $L_2$ cells produce a factor which mimics the biological activity of NGF.

To characterize this factor, $L_2$ cells were grown in the presence of the 90% MEM, 10% heat-inactivated fetal calf serum growth medium until cells had formed a confluent monolayer ($3 \times 10^7$ cells per flask). The growth medium was removed, cells were washed three times with 5 ml of MEM to remove serum, and then 20 ml of MEM without serum was added to the flask. After 10 days in culture, the medium was collected, centrifuged to remove floating cells, dialyzed exhaustively against 0.01 M ammonium acetate, and lyophilized. The residue was redissolved in MEM to a concentration 100 times the original and dialyzed against 50 ml of MEM at 4°. Sensory ganglia assays revealed that this solution possessed strong neurite-extension-promoting activity, whereas the dialysate had no effect upon ganglia. The biological activity of the supernatant was abolished upon heating at 56° for 60 min. Moreover, when 0.1 ml of concentrated supernatant solution was

treated with 0.1 ml of a 1:10 dilution of heat-inactivated (56°, 30 min) anti-NGF serum, the growth effect was also eliminated. In this regard, it should be pointed out that anti-NGF sera do not kill sensory ganglia in culture, as shown by the fact that neurite extension from sensory ganglia can be induced by $N^6$, $O^{2'}$-dibutyryl adenosine 3':5'-cyclic monophosphate (28, 29) in the presence of anti-NGF. (Unpublished observations from this laboratory; and personal communication from R. A. Murphy and F. J. Roisen.)

As shown below, $L_2$ cells produce a protein which is immunochemically similar to NGF. $L_2$ cells were grown with MEM (25 ml) without serum as described above. Samples (2.0 ml) were withdrawn after several time intervals, floating cells were removed by centrifugation, and the supernatant solutions dialyzed thoroughly against 0.01 M ammonium acetate and lyophilized. Each dry residue was redissolved in 100 μl of fresh MEM and assayed together with several NGF standard solutions by the phage method. Fig. 5 presents a plot of cumulative nanograms of NGF versus time in culture. It will be seen that the content of immunoreactive NGF in the culture fluid rises to a maximum at 4–5 days, and then falls—presumably due to degradation. From these data, we calculate that at the peak, the culture fluid contains the equivalent of 4.8 ng of NGF per ml. This result could not stem from residual calf serum in the culture, since undiluted fetal-calf serum contains no immunoreactive NGF by phage assay.

To see whether other fibroblast cell lines might also synthesize NGF, we have examined 100-fold concentrated mouse 3T3 cell supernatant solutions by immunoassay as described above for $L_2$ cells and in the *legend* to Fig. 3. Table 1 reveals that both 3T3 and SV 3T3 cells produce immunoreactive NGF. Further work is required to see whether the nerve growth factor synthesized by these cells is chemically identical to mouse submaxillary gland NGF.

## DISCUSSION

Production of NGF by L cells is pertinent to the well-established observation that L cells are a source of so-called "conditioned tissue culture medium" (26). That is, these cells produce factor(s) which stimulate the proliferation of other unrelated cells. For example, in addition to conditioned medium, L cells produce a macrophage growth factor (30) which induce macrophages to multiply in culture and a marrow-stimulating factor which enhances the uptake of thymidine by mouse marrow cells (31). To this list can now be added NGF, which also stimulates growth—but which, unlike the others, has been well characterized chemically (23). The relationship among these factors, if any, is not known, but it

TABLE 1. *Production of NGF by 3T3 and SV 3T3 cells*

| Cells | Days in culture | NGF* (ng/ml) |
|---|---|---|
| 3T3 | 3 | 0.49 |
| 3T3 | 4 | 0.68 |
| SV 3T3 | 5 | 0.51 |

Cells were grown at 37° in the presence of 90% McCoy's medium and 10% heat-inactivated fetal-calf serum for the times indicated. Supernatant solutions were analyzed by the phage method as described in the *legend* to Fig. 3.

* Values represent ng of NGF equivalents per ml of original culture supernatant solution. Each 3T3 culture flask contained $7 \times 10^6$ cells; the SV 3T3 flask contained $2 \times 10^6$ cells.

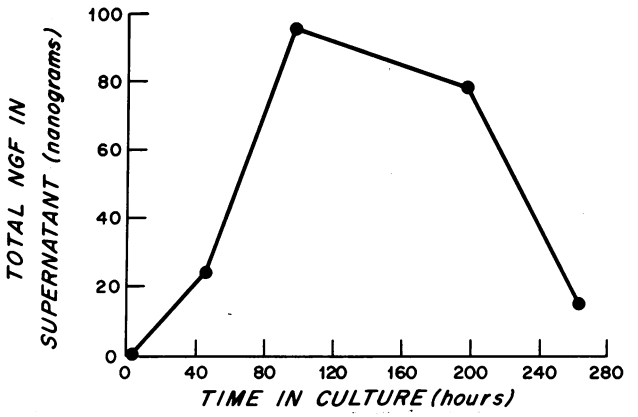


FIG. 5. Production of NGF by $L_2$ cells. A culture of $L_2$ cells was prepared and the supernatant sampled over time (see *text*). Phage assays were performed as described in the *legend* to Fig. 3.

should be possible to determine whether any of the "conditioned medium" activities are due to NGF itself.

The observation that mouse 3T3 fibroblasts produce immunoreactive NGF demonstrates that production of this growth factor is not solely a property of transformed cells. These findings support the original studies of Bueker and of Levi-Montalcini and her colleagues who showed that mouse sarcomas 180 and 37 (4–6) produce a nerve growth factor. Granuloma tissue also shows nerve-growth-factor-like activity *in vitro*, although in this case the growth factor has not been characterized chemically and the cells which produce it have not been identified. The results of the present study indicate that the NGF produced by $L_2$ cells and by 3T3 fibroblasts is similar to and possibly identical with male mouse submaxillary gland NGF. Taken together, they also suggest that the synthesis of NGF may be a general property of fibroblasts. If so, this could explain why NGF activity is found in granulation tissue and why removal of the submaxillary glands only temporarily reduces the serum concentration of NGF (11).

The observation that mouse sarcomas 37 and 180 also produce a nerve growth-promoting activity would appear to support this idea. However, we note that sarcoma 180 was originally described in 1914 as an axillary *carcinoma* of a white male mouse, and sarcoma 37 originated in 1908 as an *adenocarcinoma* of mouse mammary gland (32). Thus, the precise nature of these tumors is unclear.

Finally we note a recent study which shows that glial cells in culture release a factor which stimulates morphological differentiation of neuroblastoma cells (33). Moreover, another study has demonstrated that ganglionic nonneuronal cells can influence the growth of ganglionic neurons in culture (34). It seems possible that glial cells might also produce NGF.

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