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Nerve Growth Factor in Rat Glioma Cells

(glia/complement fixation/neuroblastoma/isoelectric focusing/development)

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ABSTRACT **We have shown that rat glioma tumors contain a protein that crossreacts with antibody against mouse 2.5S nerve growth factor prepared in rabbit in microcomplement fixation assays and has analogous isoelectric points to all the hybrids of 2.5S nerve growth factor (a dimer). Partially purified protein preparations from gliomas cause chick dorsal root ganglia to extend neurites in the nerve growth factor assay and cause morphological differentiation of mouse neuroblastoma cells. We conclude that the rat glioma protein is homologous to mouse salivary nerve growth factor and suggest the possibility that glial nerve growth factor plays a role in neuronal development and regeneration.**

Nerve growth factor (NGF) is a protein that selectively enhances growth and differentiation of sympathetic and sensory ganglia (1). The sensory and sympathetic ganglion cells respond maximally to NGF during the period in which the outgrowth of axons normally occurs. This period also corresponds with the period of glial proliferation (2). Additionally, it is well known that Schwann cells and glial cells act as guides for regenerating axons (3). These facts are consistent with a hypothesis that glia are involved in the process of neurite extension by secreting NGF, which stimulates differentiation. Establishment of a glioblastoma cell line in tissue culture provides a convenient source of homogeneous cells of glial origin and makes investigation of some aspects of this hypothesis possible (4).

Burnham *et al.* (5) have shown that ganglionic non-neuronal cells can replace a nerve growth factor requirement for the survival *in vitro* of dissociated ganglionic neurons, and Monard *et al.* (6) have recently reported that glial cells in tissue culture release a factor into their medium that induces a high degree of differentiation in neuroblastoma cells. We have confirmed the results of Monard *et al.* in our laboratory, using C49 neuroblastoma cells as an assay system. This report presents additional experiments that show that rat glioma cells contain a protein with immunological, physical, and biological properties similar to those of mouse nerve growth factor.

MATERIALS AND METHODS

Purification of 2.5S NGF. 2.5S NGF was purified from submaxillary glands of 40- to 60-day-old mice by a rapid single-column procedure described by Schenker and Shooter (1974, manuscript in preparation). The final product was stored in 50 mM Na-acetate buffer pH 4.0 at −70°.

A similar purification technique was used with rat glioma tumors, which were gifts of Dr. John Leith and Wolfgang Schilling of the Lawrence Radiation Laboratory, University of California, Berkeley. The cells had been passed in tissue culture and into the flanks of rats. The tumors (26 g) were carefully dissected from four animals and any surface blood was thoroughly washed away. None of the tumors had entered the necrotic stage.

Neuroblastoma Assay. Clone NB (neuroblastoma) C49 (our number), originally obtained from Dr. G. Sato's laboratory (UC San Diego) was cultured in Dulbecco's modified Eagle's medium buffered with bicarbonate, pH 7.4, containing 10% heat-inactivated fetal-calf serum. The cells were grown on Falcon Optilux tissue culture dishes in an atmosphere of 5% CO_2. They were transferred with minimal trypsin digestion. They were plated at low density (usually 50,000 cells per 60-mm plate) to prevent confluence before the time their response was recorded. They were allowed 24 hr to adapt before the test medium was added. The test media were sterilized through Millipore syringe filters (no. SLGS 02505), and sterile serial dilutions were made. Cells were scored positive if they had a neurite at least equal in length to the diameter of the cell [Monard *et al.* (6)].

Embryonic Chick Dorsal Root Ganglion NGF Assay. Dorsal root ganglia dissected from 8-day-old chick embryos were placed on a collagen-coated coverslip in a petri dish (4 dorsal root ganglia per plate) (7). The medium containing the assay material was modified Eagle's medium buffered with bicarbonate. The response was scored after 48 hr of incubation.

Microcomplement Fixation Technique. Antibody against mouse 2.5S-NGF prepared in rabbit was obtained from Dr. Harvey Herschman of the University of California, Los Angeles. This antibody preparation was shown to be monospecific by Ouchterlony analysis and immunoelectrophoresis (H. Herschman, personal communication). Microcomplement fixation assays were performed as described by Champion *et al.* (8), with the exception that the final volume was halved.

Polyacrylamide Isoelectric Focusing Disc Gels. The technique used is described in ref. 9, except that samples were added in 10% sucrose–1% ampholines and layered beneath 5% sucrose–1% ampholines. Gels were run for 30 min at 50 V and 2.5 hr at 100 V.

RESULTS

Microcomplement Fixation Assays. The immunological reactivity of rat glioma extract was compared to that of

Abbreviation: NGF, nerve growth factor.

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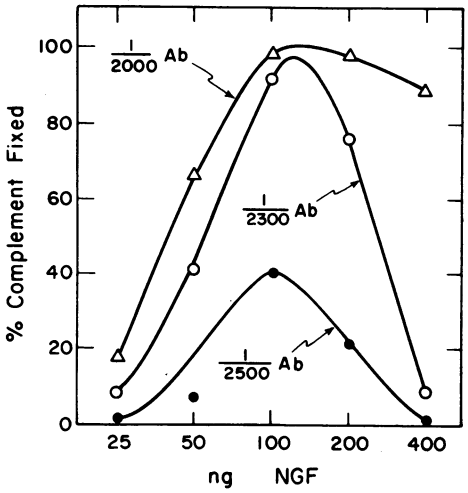

FIG. 1. Microcomplement fixation assay of mouse salivary 2.5S NGF. Antibody against mouse salivary 2.5S NGF prepared in rabbits was reacted with different concentrations of 2.5S NGF at the titers indicated in the figure in the presence of guinea pig complement and sheep erythrocytes as indicated in *Methods*. *Ab*, antibody.

2.5S NGF purified from mouse submaxillary glands. As shown in Fig. 1, 2.5S NGF purified from mouse submaxillary gland required an antiserum dilution of 1:2300 for maximal complement fixation in the assay utilized (8). Crude extracts of glioma tumors contained a substance that crossreacted in this system and reacted maximally at an antiserum dilution of 1:500 (Fig. 2*A*). Due to anticomplementarity of unknown substances in the crude glial extract, it was desirable to purify the antigen. The partially purified antigen from glioma gave characteristic microcomplement fixation curves, as shown in Fig. 2*B*. These varied as expected with antibody dilution after correction for some remaining anticomplementarity. The ratio of antibody titers required to produce maximal complement fixation in these microcomplement fixation assays can be used to measure differences in amino-acid sequence between two related antigens (10). In the experiments reported here, this ratio was close to four (2000/500) for mouse salivary NGF and rat glioma antigen, which indicates that rat glioma and mouse submaxillary NGFs differ in amino-acid sequence by approximately 10% if the relationship is valid in this case.

Dorsal Root Ganglion Assay. The classical dorsal root ganglion response was used to assay for NGF activity. A typical response was seen in the region around 200 ng of protein per ml of partially purified glioma NGF in modified Eagle's medium. Since NGF's optimal activity in this assay is typically 10 ng/ml, we roughly estimate that 5% of the partially purified protein is active NGF. This estimate corresponds roughly to the level of purity evident in isoelectric focusing gels of the preparation (see below).

Neuroblastoma Assay. The partially purified NGF causes "differentiation" of NB C49 cells grown in modified Eagle's medium plus 10% heat-inactivated fetal-calf serum in 5% CO_2, as shown in Table 1. Morphologically the cells were similar to those recently reported by Monard *et al.* (6) and to cells incubated with mouse 2.5S NGF.

Isoelectric Focusing in Polyacrylamide Disc Gels. A sample of basic protein partially purified from glioma tumors according to *Methods* was analyzed in isoelectric focusing gels. The results are shown in Fig. 3. The partially purified glioma sample had three protein bands visible whose isoelectric points were indistinguishable from the three mouse 2.5S NGF hybrid bands. These three bands reflect the fact that 2.5S NGF is a dimer of 26,500 molecular weight (11) whose 13,250 molecular weight chains can lose a COOH-terminal arginine, which decreases their isoelectric points (12). The three bands represent a dimer of native NGF chains, a hybrid containing one native chain and one desarginine chain, and a dimer of desarginine chains (12).

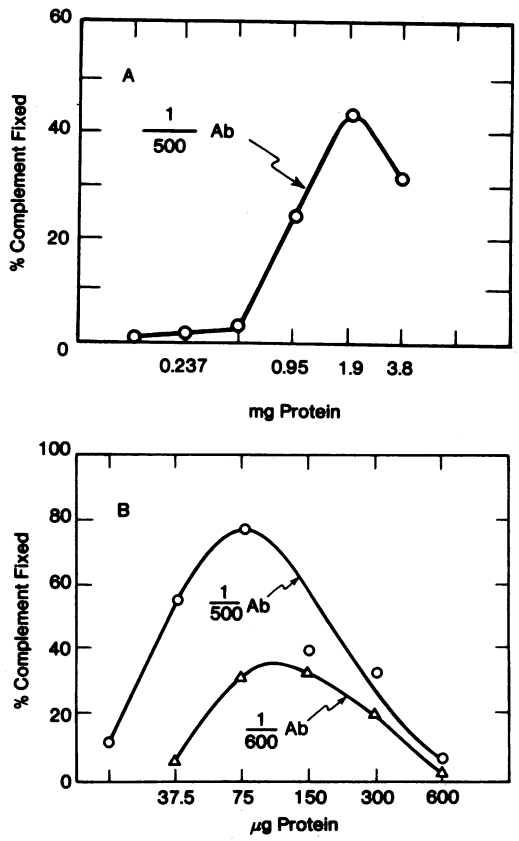

FIG. 2. (*A*) Microcomplement fixation assay of crude rat glioma extract using rabbit anti-mouse salivary 2.5S NGF. Assays were conducted as indicated in legend of Fig. 1 and in *Methods*. (*B*) Microcomplement fixation assay of the basic protein fraction from rat glioma extracts purified as indicated in *Methods*.

TABLE 1. *Morphological differentiation of neuroblastoma C49*

Media tested	% Neuroblastoma with processes
10% fetal-calf serum	18
No fetal-calf serum	55
10% fetal-calf serum conditioned by rat C-6 glial cells	78
10% fetal-calf serum + glioma NGF fraction (25 μg/ml)	88
10% fetal-calf serum + glioma NGF fraction (2 μg/nl)	68

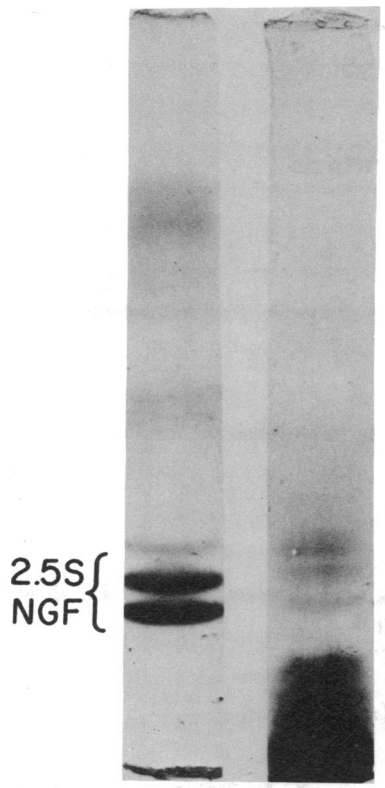

FIG. 3. Comparison of isoelectric focusing gels [pH 3 (*top*)–10] of mouse salivary 2.5S NGF (*left*) and rat glioma basic protein fraction (*right*). Procedure is described in *Methods*.

DISCUSSION

Our results demonstrate that rat glioma cells contain a basic protein with immunological and physical properties similar to mouse salivary 2.5S NGF. These similarities lead us to conclude that this protein is the rat homolog of the well-characterized mouse 2.5S NGF. The biological activities of our glioma protein preparation are also very similar to those of mouse NGF. However, since the biological assays used are responsive to substances other than NGF, definitive proof of biological identity must await purification of the glioma NGF to homogeneity.

The biological importance of NGF in development has been demonstrated in studies where injection of antibody against NGF into newborn mice prevents development of the sympathetic nervous system and, hence, is called an immunosympathectomy (1). NGF is present in high levels in submaxillary glands of mice only after puberty, and removal of these glands from newborn animals has no effect on sympathetic development (13), suggesting that an alternative site of synthesis must be involved in this development. The identification of NGF in cells of glial origin strengthens the possibility that the developmentally important site of NGF synthesis is glial cells. Both the temporal aspects, that is, glial proliferation during the period of neurite extension, and the spatial aspect of glial development encouraged our exploration of the possibility that glia synthesize and secrete NGF which then may act as a "pleiotypic" stimulant for the neuron. Glia have also been observed to play an active role in neural regeneration (3). NGF has been shown to stimulate regeneration in central noradrenergic, brain monoamine, spinal, and sympathetic nerve fibers. Therefore, regeneration may also involve stimulation of neurons by NGF of glial origin.

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