

NERVE GROWTH FACTOR PREVENTS THE DEATH AND STIMULATES THE NEURONAL DIFFERENTIATION OF CLONAL PC12 PHEOCHROMOCYTOMA CELLS IN SERUM-FREE MEDIUM

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ABSTRACT

The PC12 clone is a noradrenergic cell line derived from a rat pheochromocytoma. In culture medium containing horse serum, PC12 cells undergo mitosis; when nerve growth factor (NGF) is included in the medium, the cells cease multiplication and extend neurites. It is shown here: (a) that PC12 cells are not viable in serum-free medium. When serum is withdrawn, 90% of the cells die within 4–6 days and 99% by 2–3 wk. (b) If NGF is added at the time of serum withdrawal, the cells undergo one doubling and remain viable for at least 1 mo. (c) Addition of NGF to cultures after more than 2 days in serum-free conditions results in maintenance of surviving cells, but not in an increase in cell number. (d) NGF also induces neurite outgrowth from PC12 cells in serum-free medium. (e) NGF-treated cells exhibit much less cell-cell and neurite-neurite aggregation in the absence than in the presence of serum. (f) The apparent minimum level of 2.5S NGF required for PC12 survival and morphological differentiation in serum-free medium is about 10 ng/ml (~0.4 nM). (g) Withdrawal of NGF in serum-free conditions results in degeneration of neurites and loss of cell viability. (h) Experiments with camptothecin demonstrate that the effects of NGF on survival and neurite outgrowth may be uncoupled and suggest that the survival effects are transcriptionally independent. The present results also suggest that PC12 cells have a requirement for NGF (similar to that of normal sympathetic neurons) and that serum may substitute for this requirement. In addition, the present system of maintaining a highly differentiated cell line in a chemically defined medium suggests certain experimental opportunities.

KEY WORDS nerve growth factor ·
differentiation · pheochromocytoma · PC12
cell line · serum-free medium · neurites

Nerve growth factor protein (NGF) is known to have a number of effects on responsive sympa-

thetic and sensory ganglionic neurons (for reviews, see references 18, 30, and 34). These effects include stimulation of neurite outgrowth (18), increase in cell size (18) and induction of certain enzymes involved in neurotransmitter synthesis (26). In addition, NGF also appears to play a role

in supporting the survival of such neurons. For example, sympathetic and responsive sensory neurons fail to survive and extend neurites *in vitro* unless exogenous NGF is added to the culture medium (5, 16). Also, treatment of neonates with anti-serum to NGF results in the near-total loss of their sympathetic neurons (17).

Several studies have shown that cultured adrenal medullary pheochromocytoma cells can also respond to NGF by outgrowth of neuron-like processes (27, 28). A clonal cell line known as PC12 has been established (8) from a transplantable rat pheochromocytoma (32), and this line has been particularly promising as a model with which to study the mechanism(s) of action of NGF. In culture medium containing horse serum but not NGF, PC12 cells undergo mitosis (8) and display a number of properties of adrenal chromaffin cells, such as the presence of intracellular chromaffin granules and the abilities to synthesize, store, take up, and release catecholamines (8, 9, 11, 13). The cells also synthesize, store, and release acetylcholine (10, 24). Under such conditions, PC12 cells do not extend neurites (8). Within a few days after nanogram levels of NGF are added to the culture medium, PC12 cells cease mitosis, extend long, branching neurites (8), become electrically excitable and exhibit a large increase in chemosensitivity to acetylcholine (7). Such effects are reversible. Within several days after withdrawal of NGF from PC12 cultures, the neurites disintegrate and mitotic activity recommences (8). Such studies have suggested that, in response to NGF treatment, PC12 cells take on many of the properties of normal sympathetic neurons (7, 8).

One particular contrast of PC12 cells with responsive normal neurons is that while the pheochromocytoma cells respond to NGF, they do not appear to require it for their survival in serum-containing medium. In the present study, however, we demonstrate that PC12 cells are not viable in serum-free medium and that under such conditions NGF prevents cell death and promotes neurite outgrowth.

MATERIALS AND METHODS

Cell Culture

PC12 rat pheochromocytoma cells (250–350 generations after isolation) were maintained in cell culture as previously described (8, 9). For subculture, cells were mechanically dislodged from the dish by trituration. For

all experiments, cells (in 1.5 ml of medium) were plated onto 35-mm tissue culture dishes which had been previously coated with rat tail collagen (8, 9). Culture medium was exchanged three times weekly. Mouse 2.5S nerve growth factor was prepared according to the method of Bocchini and Angeletti (4). In some experiments, the culture medium was supplemented with 1 $\mu\text{g}/\text{ml}$ camptothecin (kindly supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute).

Cell Counts and Determination of Proportion of Process-Bearing Cells

Cell number per culture was determined by either of two means. In most cases, the culture medium was removed and the cells were brought into suspension by treatment with trypsin (0.1% DIFCO 1:250, for 15–30 min at 37°C) and counted in a hemacytometer. In some experiments, cell number was determined by strip counts (31) of approx. 2% of the surface area of the dish. Application of both methods to the same cultures gave values which agreed to within $\pm 10\%$. For each sample analyzed, at least 1,000 cells were counted. In several experiments, viability was confirmed by the use of trypan blue (>95% of cells counted excluded the dye).

Proportions of process-bearing cells per culture were determined by strip counts as previously described (8, 31). Processes were scored only if their length was at least twice the cell body diameter (>20 μm in length). At least 300 cells were scored per dish.

RESULTS

Serum Requirement for Survival of PC12 Cells in the Absence of NGF

Under their previously described growth conditions (8) in serum-containing medium (85% RPMI 1640, 5% fetal calf serum, 10% horse serum, and no NGF), PC12 cells show excellent viability and growth (Fig. 1). In order to observe the effect of serum-free conditions on PC12 cells, the growth medium was removed, the cultures were washed three times with serum-free medium (RPMI 1640), and then either (a) the cultures were re-fed with serum-free medium, or (b) the cells were detached and subcultured on new dishes in serum-free medium, or (c) the cells were maintained for 1–5 days in serum-free medium and then subcultured on new dishes in serum-free medium. In each case, exposure to medium free of serum and of NGF resulted in immediate loss of cell viability (Figs 1, 6, and 7). In five separate experiments, approx. 90% of the cells died within 4–6 days and approx. 99% within 2–3 wk. Such

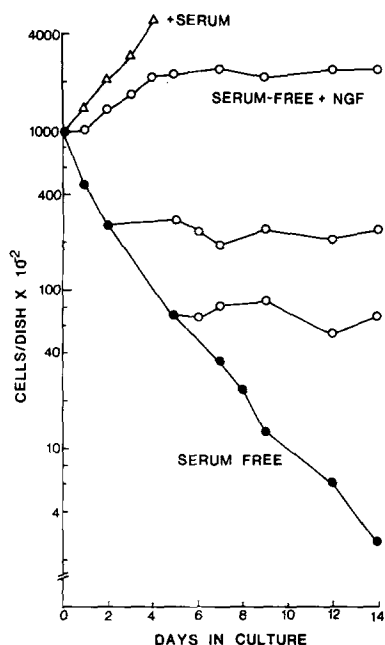


FIGURE 1 Effect of NGF on survival of PC12 cells in serum-free medium. Sister cultures were maintained with serum-containing medium until day zero. The cultures were then washed three times with serum-free medium and fed with either serum-containing medium (triangles), serum-free medium (closed circles) or serum-free medium plus NGF (open circles). On days 2 and 5, some cultures were changed from serum-free medium to serum-free medium plus NGF. Cell counts are averages of determinations on duplicate cultures.

effects occurred at initial cell densities ranging from very sparse to very crowded (i.e. 10^5 - 10^7 cells/35-mm dish). Cell death was accompanied by cell disintegration, which in turn was reflected by the appearance of membrane fragments and insoluble debris in the culture medium. Although PC12 cells are viable in suspension, living cells were not observed floating in the medium of serum-free cultures and, hence, loss of cell number was not due to cell detachment from the substrate. Also, in contrast to observations reported for clonal murine C1300 neuroblastoma cells (25), withdrawal of serum from PC12 cultures did not induce neurite outgrowth.

Effect of NGF on Viability and Morphologic Differentiation of PC12 Cells in Serum-Free Medium

Addition of 2.5S NGF (50 ng/ml or ~2 nM, unless otherwise specified) to serum-free cultures

produced profound effects on PC12 cell viability. When NGF was added at the time of change-over to serum-free conditions (see Fig. 1 for the results of one such experiment), the cell number underwent approximately one doubling and then remained stable for at least 6 wk. When NGF was added to cultures after several days of treatment with serum-free medium alone, the loss of cells immediately ceased, and the population remained at near-constant levels (Fig. 1). Cell number did not increase, however, when NGF was added to cultures that had been previously exposed to serum-free medium for 2-7 days. In all experiments, examination of the cultures after addition of NGF revealed no evidence of continued cell death (i.e., there was no debris of the type present in serum-free cultures maintained without NGF). This suggests that maintenance of cell number by NGF was due to continued cell viability rather than to an equilibrium between cell death and cell division.

In addition to preventing the death of PC12 cells in serum-free medium, NGF also induced their morphological differentiation. Fig. 2 (see also Fig. 7) shows the rate of appearance of neurites in serum-free cultures after addition of NGF. Note that the lag time for first appearance of neurites was substantially decreased in cultures which were exposed to serum-free medium for

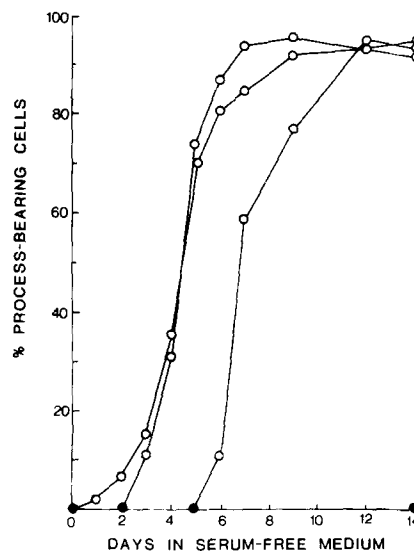


FIGURE 2 Rate of appearance of neurites in serum-free cultures of PC12 cells maintained in the presence (open circles) or absence (closed circles) of NGF. Culture conditions are as described in legend of Fig. 1.

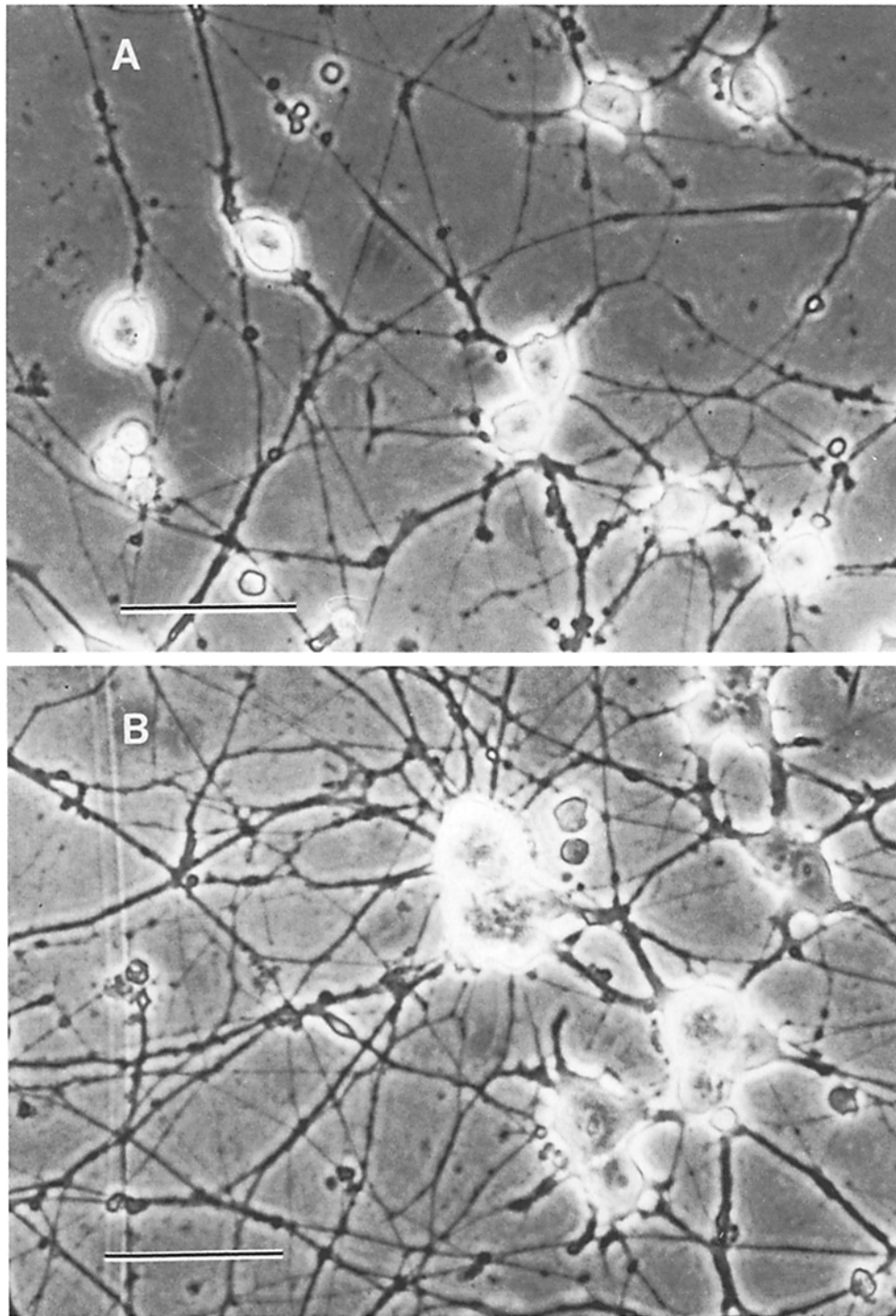


FIGURE 3 Phase-contrast micrographs of living PC12 cells treated with NGF for 14 days in (A) the absence or (B) presence of serum. Bar, 100 μm . $\times 250$.

several days before addition of the factor. The morphology of PC12 cells treated with NGF in the absence of serum (Fig. 3A) was in many ways comparable to that of cultured primary sympathetic neurons (cf. reference 20). The cell bodies extended long (>100 μm) neurites which were varicose and which showed typical growth-cone-like extensions. However, in contrast to sympathetic neurons and to PC12 cells treated with NGF in the presence of serum (Fig. 3B), the neurites of cells in serum-free medium were finer and showed much less formation of small bundles or fascicles. Also, the cell bodies of NGF-treated cells showed appreciably less tendency to aggregate in serum-free cultures and formed more of a monolayer than in the presence of serum.

The effect of NGF in serum-free medium was also tested on several other cell lines which do not show evident responses to the factor in the presence of serum. These lines included C₆ glioma cells (3), T28 neuroblastoma X sympathetic ganglion cell hybrids (12), and an NGF-unresponsive rat cell line developed by us and designated as F-4. In each case, NGF did not

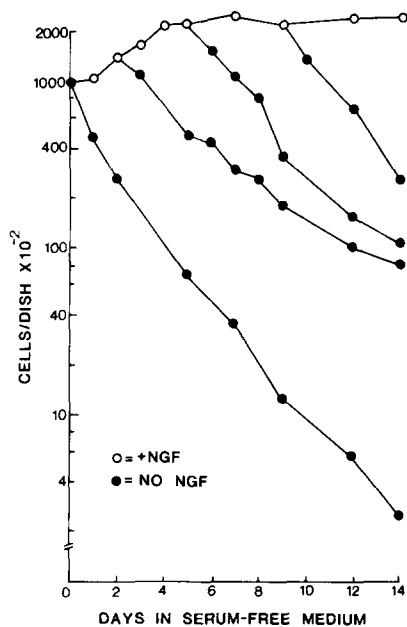


FIGURE 4 Reversibility of the effect of NGF on PC12 cell survival in serum-free medium. Culture conditions were as indicated in legend of Fig. 1. On days 2, 5, and 9, NGF was withdrawn from certain cultures as indicated. This was achieved by washing (three times) and refeeding the cultures with medium free of both NGF and serum.

affect either cell viability or morphology in the absence of serum. Thus, NGF does not appear to be a general survival factor for all cell lines.

Reversibility of the Effect of NGF on Survival and Differentiation of PC12 Cells in the Absence of Serum

When NGF was withdrawn from serum-free cultures of PC12 cells after various times of treatment, cells began to die within 24 h (Fig. 4). In addition, removal of NGF also resulted in loss of neurites by disintegration. As shown in Fig. 5, this loss of processes was even more rapid than loss of cell number.

Dose-Response Relationship for the Effects of NGF in Serum-Free Cultures

PC12 cells were exposed to serum-free medium for 2 days, and then equal numbers of cells were subcultured in the presence of various levels of 2.5S NGF. After 1 wk of treatment, both cell number and proportion of process-bearing cells were determined for each culture. As shown in Fig. 6, the minimal level of NGF required for both maximal maintenance of survival and morphologic differentiation was on the order of 10 ng/ml (~ 0.4 nM). This corresponds to approx. 1

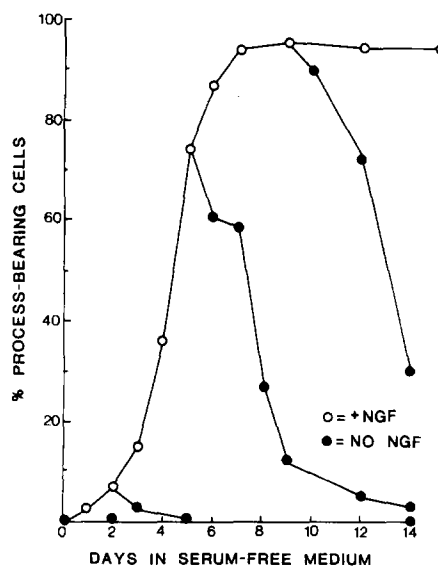


FIGURE 5 Reversibility of the effect of NGF on PC12 cell survival and morphological differentiation in serum-free medium. Experimental conditions are as indicated in legends of Figs. 1 and 4.

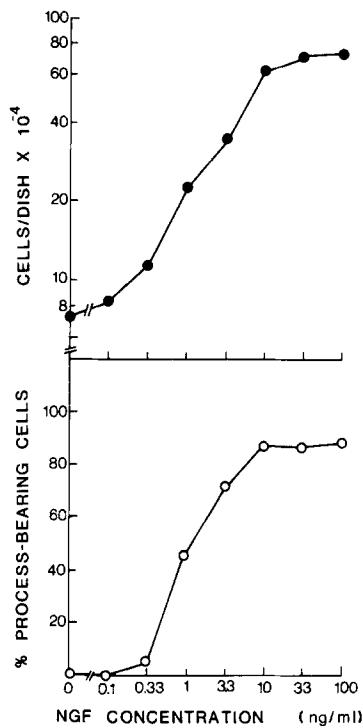


FIGURE 6 Dose-response relationship for the effects of NGF on survival (upper panel; closed circles) and morphological differentiation (lower panel; open circles) of PC12 cells in serum-free medium. Serum-free sister cultures were prepared by subculturing cells which had been previously maintained in serum-free medium for 2 days. Pairs of cultures were then treated with various levels of NGF. After 7 days, the cultures were analyzed for proportion of process-bearing cells and for cell number. Data are averages of determinations on duplicate plates.

Biological Unit/ml as defined by the classical biological assay for NGF (18). However, since NGF is known to readily adsorb to surfaces (23), particularly in the absence of carrier proteins (such as those in serum), the above figures are probably an overestimate of the true minimal level of NGF required for survival and morphologic differentiation of PC12 cells in the absence of serum.

Dissociation of the Effects of NGF on PC12 Cell Survival and Neurite Outgrowth in the Absence of Serum

Camptothecin is a drug which is known to reversibly block synthesis of messenger and ribosomal RNA in a variety of mammalian cell types

(cf. references 1, 15, and 33). Experiments with PC12 cells maintained in the presence of serum have revealed that low levels of camptothecin can block NGF-induced neurite outgrowth without affecting cell viability (D. Burstein and L. A. Greene. Manuscript in press), as well as block NGF-stimulated synthesis of certain cell proteins (21). It was therefore of interest to observe the effects of this drug on PC12 cells treated with NGF in the absence of serum. As shown in Fig. 7, treatment of PC12 cells with camptothecin (1 $\mu\text{g/ml}$) in serum-free medium did not retard cell loss in the absence of NGF. Moreover, the drug did not affect the ability of NGF to maintain cell viability in serum-free medium for at least 1 wk. However, as shown in Fig. 7, in

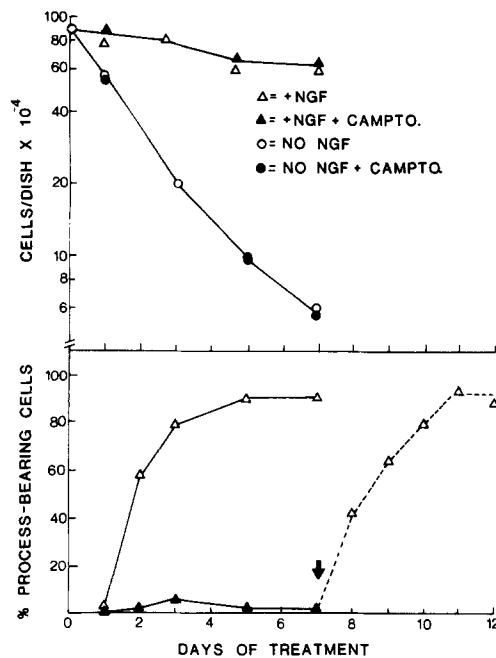


FIGURE 7 Effect of camptothecin (1 $\mu\text{g/ml}$) on NGF-induced survival (upper frame) and morphological differentiation (lower frame) of PC12 cells in serum-free medium. Serum-free sister cultures were prepared by subculturing cells which had been previously maintained in serum-free medium for 2 days. Cultures were treated with either NGF (open triangles), NGF plus camptothecin (closed triangles), camptothecin (closed circles), or no additives (open circles). After various lengths of time, as indicated, duplicate cultures were assayed for proportion of process-bearing cells and for cell number. At the time indicated by the arrow, the medium of cultures containing both NGF and camptothecin was replaced with medium containing only NGF. Data are averages of determinations on duplicate plates.

contrast to cells which were treated with NGF alone, those also treated with camptothecin failed to extend processes. As a control for cell viability and function, NGF-induced neurite outgrowth commenced when the camptothecin was removed from the culture medium. Such findings suggest in part that it is possible to uncouple the effects of NGF on PC12 cell survival from those on morphologic differentiation.

DISCUSSION

With respect to their dependence on serum and on NGF, PC12 cells are, in some respects, similar to and yet different from normal sympathetic neurons. In the presence of horse serum (but not of fetal calf serum), PC12 cells survive and multiply in the absence of exogenously supplied NGF. Under such culture conditions, PC12 cells can, however, respond to NGF by cessation of cell division and neurite outgrowth (8). It has been shown here that PC12 cells do not survive in serum-free medium, but that they will do so when NGF is present. In contrast, normal sympathetic neurons require NGF for their survival when plated *in vitro*, even in the presence of serum (5, 16). Moreover, sympathetic and responsive sensory neurons can also be maintained *in vitro* (at least for limited periods of time) by NGF under serum-free conditions (14, 19, 20).

The ability of serum-treated PC12 cells (in contrast with normal sympathetic neurons) to survive and divide in the absence of NGF is not surprising in light of their being tumor cells. PC12 cells thus appear to be supported by serum factor(s) which cannot maintain survival of young postmitotic sympathetic neurons. From this point of view, it may not be that NGF can substitute for the PC12 cell serum requirement, but rather that serum can substitute for the PC12 cell NGF requirement. Hence, when serum is removed, PC12 cells show the same requirement for NGF as do normal sympathetic neurons. The components of serum responsible for PC12 cell survival and growth appear to be neither universal nor nonspecific, since PC12 cultures cannot be supported by fetal calf serum (8). Furthermore, such serum factors do not appear to be identical to NGF. For example, the level of horse serum which maintains PC12 cultures does not stimulate neurite outgrowth from PC12 cells or survival of sympathetic neurons. Also, anti-NGF does not affect viability in serum-containing PC12

cultures (P. Calissano and L. A. Greene, unpublished observations).

It has been hypothesized that PC12 cells may be analogous to a developmental precursor of normal sympathetic neurons (7, 8, 28). In this regard, the behavior of PC12 cells may resemble that of *in vivo* embryonic cells. In particular, recent evidence suggests that catecholamine-containing sympathetic ganglion cells maintained *in vitro* from certain embryonic stages can respond to NGF, but do not require it for survival (6).

The mechanism(s) by which NGF maintains the viability of PC12 cells in the absence of serum is not presently clear. The present findings, however, do appear to exclude several possibilities. First, it is evident that this phenomenon does not require ancillary factors present in serum such as hormones, steroids, or macromolecules. Second, since PC12 cells may undergo at least one doubling when NGF is introduced along with serum-free medium, the survival effect of the factor is not due to trapping of the cells in a "protected" part of the cell cycle. Third, survival does not appear to be a consequence of neurite outgrowth. The effect of NGF on PC12 cell viability occurs at times well before neurite outgrowth is evident. Moreover, NGF maintains survival even when neurite outgrowth is blocked by treatment with camptothecin.

In the present experiments with serum-free medium, both survival and neuronal differentiation of PC12 cells exhibited similar dose-response curves with respect to NGF concentration (Fig. 6). This suggests that these two effects of NGF may be mediated via shared initial steps. Among these could be interaction of NGF with a common receptor site. In this regard, it is of interest that the apparent K_D (0.2–0.3 nM) for binding of NGF with a receptor on mammalian ganglionic membranes (2) is comparable in magnitude to the minimally required levels of NGF in the present experiments. On the other hand, the observation that camptothecin uncouples the effects of NGF on survival and neurite outgrowth implies that subsequent steps in the mediation of these two effects may follow independent or divergent pathways.

The present experiments employing camptothecin raise an additional point regarding the molecular level at which NGF promotes cell survival. Camptothecin has been shown in several cell systems to interfere selectively with synthesis of messenger and ribosomal RNA (1, 15, 33), and

in PC12 cultures (in the presence of serum) to block NGF-induced neurite outgrowth (D. Burstein and L. Greene. Manuscript in press) and selective induction of synthesis of several proteins (21). Such findings suggest that the survival-promoting effect of NGF is independent of RNA transcription. This notion is consistent with the previous observation that NGF promotes survival of cultured chick embryo ganglionic neurons even in the presence of levels of actinomycin D which block greater than 90% of incorporation of radiolabeled uridine into TCA-precipitable material (22). Confirmation of this point in the present system, however, awaits a detailed analysis of the effect of camptothecin on PC12 cell RNA synthesis.

Aside from supporting PC12 cell viability, a second major effect of NGF described here was to promote neurite outgrowth in a chemically defined medium. This observation raises the point that initiation and long-term maintenance of NGF-promoted fiber outgrowth from PC12 cells does not require a continuous exogenous supply of additional factors such as steroids, peptide hormones, or macromolecules or of potential membrane components found in serum such as complex lipids. Another interesting aspect of PC12 cultures maintained with NGF in the absence of serum was that the cells display finer neurites, near-absence of fiber fasciculation, and much less tendency to form cell-cell aggregates than do cells in NGF-treated cultures which contain serum. Such observations suggest that the effect of NGF on morphology and cell-cell interaction in PC12 cultures may be profoundly influenced by environmental factors such as serum. Furthermore, in preliminary experiments we have noted similar morphologic effects in serum-free cultures of normal sympathetic neurons, while Ludueña (19) has reported that neurons in serum-free cultures of chick embryo sensory ganglia display alterations in morphology and interaction with substrate. Thus, the influence of serum on cell interactions of NGF-responsive cells is apparently not limited to the PC12 line.

One last aspect which merits brief discussion is the experimental implications of the observation that clonal PC12 cells may be maintained in serum-free medium. Among the potential uses of this system is to study in a chemically defined environment (a) the mechanism(s) by which NGF promotes cell survival; (b) the effects of addition of known amounts of defined substances on var-

ious differentiated neuronal properties; (c) the effects of serum and serum components on cell morphology and cell-cell interaction; (d) the testing and identification of serum components which can substitute for the NGF requirement of a neoplastic cell line.

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