Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor

(sympathetic neurons/cell culture/catecholamines/differentiation/neurites)

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ABSTRACT A single cell clonal line which responds reversibly to nerve growth factor (NGF) has been established from a transplantable rat adrenal pheochromocytoma. This line, designated PC12, has a homogeneous and near-diploid chromosome number of 40. By 1 week’s exposure to NGF, PC12 ceases to grow and begin to differentiate and produce large quantities of noradrenergic properties. Such clonal PC12 lines have been used to study NGF expression and store the peptide in the granule, which in turn, can release it as a neurotransmitter. The PC12 cell line should be a useful model system for neurobiological and neurochemical studies.

Clonal cell lines which express neuronal properties are useful model systems for studying the nervous system at the single cell and molecular levels. Such lines have been established from human and murine neuroblastomas (1, 2) and from rat central nervous system tumors (3).

Warren, DeLellis et al. (4, 5) have reported the induction and partial characterization of a transplantable rat pheochromocytoma which expressed the differentiated properties of adrenal chromaffin cells in vitro. Recently, we reported (6) that chromaffin granule-containing cells cultured from this tumor responded to treatment with nerve growth factor protein (NGF) (7–9) by extending long, branching neuronal-like processes. In the present communication, we describe the establishment and properties of a clonal line of pheochromocytoma cells which respond to NGF and which synthesize and store catecholamines.

MATERIALS AND METHODS

Culture Methods and Media. Pheochromocytoma cells were obtained from a solid tumor passed subcutaneously in New England Deaconess Hospital strain white rats. (Tumor-bearing rats were originally made available to us through the generosity of Drs. R. Perlman and S. Warren, Mr. M. Chalfie, and Ms. R. Chute.) The tumor was mechanically dissociated by mincing, followed by trituration in a pasteur pipette. The cells were subjected to three cycles of washing (with phosphate-buffered saline) and pelleting (500 X g for 5 min) in order to free them from cell debris, and were resuspended in growth medium and plated on plastic tissue culture dishes (Falcon Plastics). The following day, the lightly-adhering pheochromocytoma cells were mechanically dislodged from the plates by forceful aspiration and expulsion of the medium with a Pasteur pipette, and replated on culture dishes which were coated with rat tail collagen (10). The cells were subsequently passaged two more times on collagen-coated culture dishes and three more times on plastic culture dishes. This strategy was employed for two reasons. First, newly dissociated pheochromocytoma cells adhered very poorly to plastic culture dishes. After several passages on collagen-coated dishes (to which the cells more firmly attached), the cells had adapted to culture and could be passaged onto plastic dishes. Second, the dissociated tumors contained, in addition to the cells of interest, other cell types which grew much more rapidly than the pheochromocytoma cells and which outgrew the cultures. Such cells adhered very firmly to plastic and collagen-coated substrates and tended to be left behind on the culture dishes after mechanical dislodgement. By the means described, the slowly growing pheochromocytoma cells could be adapted to culture without being overgrown.

Growth medium consisted of 85% RPMI 1640 (11), 10% heat-inactivated horse serum, 5% fetal calf serum, 50 units/ml of penicillin, and 25 µg/ml of streptomycin. Nerve growth factor protein (2.5 S) was prepared from mouse salivary glands according to the method of Bocchini and Angeletti (8) and had an activity of 0.3 ng/unit as assayed by a dissociated cell culture bioassay (12). Unless otherwise noted, all experiments were performed with cells grown on collagen-coated tissue culture dishes.

Characterization of Cells. Previously reported assays were used to measure the activities of tyrosine hydroxylase (tyrosine 3-monoxygenase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2) (13); dopa decarboxylase (aromatic-L-amino acid decarboxylase; aromatic-L-amino-acid carboxy-lyase, EC 4.1.1.28) (ref. 14); dopamine-β-hydroxylase (dopamine β-monoxygenase; 3,4 dihydroxyphenylalanine, EC 1.14.17.1) (ref. 15); phenylethanolamine N-methyltransferase (noradrenalin N-methyltransferase; Sadenosyl-L-methionine: phenylethanolamine N-methyltransferase, EC 2.1.1.28) (ref. 16); monoamine oxidase (amine oxidase (flavin-containing); amine:oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4) (ref 17); catechol-O-methyltransferase (catechol methyltransferase; S-adenosyl-L-methionine: catechol-O-methyltransferase, EC 2.1.1.6) (ref. 18). The levels of endogenous catecholamines were measured by the trihydroxyindole-fluorometric technique (19),

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on a butanol extraction of the tissue or cells. Internal standards were used to calculate and to correct for the % recovery of amines (79%). Specific activities were calculated both per cell (based on cell counts) and per mg of total cell protein (present in tissue or culture homogenates). The latter was determined by the method of Lowry et al. (20) with use of a bovine serum albumin standard.

Karyotype analyses were performed on chromosomes which were prepared as described by Earley (21) and then banded with Gurr's Giemsa (Bio/medical Specialties (ref. 22). Cells were fixed for electron microscopy by addition of 24.6% unbuffered gluteraldehyde (TAAB Laboratories, Reading, England) to the tissue culture medium over 10–15 sec at room temperature to a final concentration of 3% (method developed by Dr. S. Landis) and were post-fixed at room temperature in 1% OsO4 in 0.1% phosphate buffer at pH 7.3. Thin sections were stained with aqueous uranyl acetate followed by lead citrate.

RESULTS

Establishment of Clonal Line. Pheochromocytoma cells were passaged as described above in order to adapt them to cell culture. Cells were then dislodged mechanically, dissociated with trypsin (0.1%; DIFCO 1:250), and plated at low density (10^4/100 mm dish). After approximately 2 months, 38 colony-clones were isolated with the use of cloning cylinders (23). One of these was propagated for approximately 20 generations and then passed for recloning (1000 cells per 100 mm dish). Seventy-five days after plating, a single colony was observed on one dish; it arose from one of a number of dispersed single cells whose location had been previously marked. This clone has been propagated (presently for about 70 generations) and is designated as PC12.

The karyotypic properties of the PC12 cells were assessed at about 45 generations following their isolation. Each of one hundred metaphases counted contained 40 chromosomes. The chromosomes consisted of 38 autosomes (two of which were large acrocentric markers) and an XY pair.

Growth Properties and NGF Response of PC12 Clonal Line. In growth medium, the PC12 cells have a round or polygonal shape and tend to grow in small clumps (Fig. 1A). The cells do not extend processes nor can they be induced to do so by means used to elicit neurite outgrowth from cultured neurenhoblastoma cells (2), i.e., exposure to serum-poor and serum-free media, dibutyryl-adenosine 3':5'-cyclic monophosphate (1 mM), actinomycin-D (2-20 μg/ml), or cytosine arabinoside (1 μM). The apparent doubling time of the PC12 cells is long—about 92 hr (Fig. 2A). Cell growth was slightly less satisfactory when medium RPMI 1640 was replaced with Dulbecco's Modified Eagle's Medium (11) and unsatisfactory when replaced with media F-12 (11), F-14 (24), CMRL 1066 (11), or McCoy's 5A (11), or when horse serum was omitted.

The PC12 cells retained their tumorigenic properties. Subcutaneous injection of the cells (5 x 10^6 to 5 x 10^7) into New England Deaconess Hospital strain rats produced tumor nodules at the site of inoculation within 20–40 days. Age (5 days to 2½ years), sex, or pregnancy of the recipients did not affect the histologic appearance of the tumors, which showed no neumatous areas. There were no metastases.

The PC12 cells are sensitive to nerve growth factor protein. Like primary sympathetic neurons (12, 25, 26), NGF-treated PC12 cells adhere poorly to plastic tissue culture dishes and well to collagen-coated substrates. By 7 days of exposure to NGF (50 ng/ml) cell multiplication ceases (Fig. 2A). At about the same time of treatment, neuronal-like processes are observed in the culture. The number, length, and density of such processes continue to increase over the next 2 weeks of treatment, until at least 80% of the cells have responded (Fig. 2B). Morpholog-

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**Fig. 1.** (A–D) Phase contrast micrographs of unfixed PC12 cells that were cultured in the absence of NGF (A), in the presence of NGF for 14 (B) and 22 (C) days, and for 1 day in the absence of NGF following 16 days in the presence of NGF (D). Bar represents 100 μm. (E–F) Electron micrographs of PC12 cells cultured in the presence of NGF for 14 days. (E) Different granule types in cell bodies of adjacent cells; (F) fascicle of two processes showing small vesicles, dense core granules, and vacuosity. Bars represent 500 (E) and 1000 (F) nm.
ically, the fibers extended by PC12 cells greatly resemble those produced by cultured primary sympathetic neurons (25, 26); that is, they are long (reaching 500–1000 µm), branch profusely, have numerous varicosities, are very fine, and form fascicles (Fig. 1B and C).

The effects of NGF on the PC12 cells are reversible. About 3 % of the cells lose their processes 24 hr after their return to NGF-free medium (Fig. 2B). Degeneration, rather than withdrawal, appears to account for this phenomenon (Fig. 1D). Removal of NGF does not, however, appear to affect the integrity of the cell bodies. Cell multiplication resumes within 3 days after removal of NGF and returns to control rates shortly thereafter (Fig. 2A). When process-bearing PC12 cells are removed from their culture dishes and replated in NGF-free media, they do not regenerate their fibers. In contrast, when such cells are replated in NGF-containing media, they begin to regenerate processes within 24 hr.

Ultrastructure. The cell bodies of the PC12 cells grown with or without NGF contain round, ovoid or somewhat irregular dense core granules, 40–350 nm in greatest dimension (Fig. 1E). In the rat, such granules have been described in the cytoplasm of adrenal chromaffin cells (50–350 nm) (refs. 27, 28); small, intensely fluorescent cells of sympathetic ganglia (50–200 nm) (refs. 29, 30); principal sympathetic neurons (70–110 nm) (refs. 29, 30), and primitive sympathetic cells (90–100 nm) (refs. 29, 30). As in cells from the uncloned tumor (5), some of the granules in the PC12 cells are homogeneously and intensely electron dense, whereas others are lighter and more granular. In addition to such granules, NGF-treated PC12 cells contain small, round vesicles that are 20–70 nm in diameter (Fig. 1F) and that generally appear to be clear, but which are sometimes seen with dense cores. These vesicles are occasionally observed in the cell bodies, but are seen more frequently in aggregates which are found in varicosities or endings of processes intermingled with larger dense core granules. Such admixtures have been demonstrated in noradrenergic neurons after glutaraldehyde-osmium fixation (31–33), and also in the presumably cholinergic endings of afferent fibers on chromaffin cells in the adrenal medulla (34).

Catecholamine Metabolism. Histochemical examination of the PC12 cells by the Falck-Hillarp technique (35, 36) reveals an intense yellow-green fluorescence which indicates the presence of stored catecholamines. Consequently, the PC12 cells (NGF-treated and untreated) were analyzed for their contents of various catecholamine neurotransmitters as well as for various enzymes involved in catecholamine metabolism. The results of these assays are summarized in Table 1 along with comparative data obtained from homogenates of the uncloned tumor and of whole rat adrenal glands. For PC12 cells, the specific activities of the synthetic enzymes tyrosine hydroxylase, dopa decarboxylase, and dopamine β-hydroxylase, as well as those of the degradative enzymes (monoamine oxidase and catechol-O-methyltransferase) were all similar to or higher in magnitude than those found for adrenal glands. The PC12 cells also contained considerable amounts of norepinephrine and dopamine. In contrast to adrenals, however, dopamine was predominant. Phenylethanolamine-N-methyltransferase (PNMT), which catalyzes the conversion of norepinephrine to epinephrine, was not detectable in the PC12 cells, nor was epinephrine itself. Many rat adrenal chromaffin cells have been shown to contain phenylethanolamine-N-methyltransferase (and are consequently adrenergic) while others do not (and are consequently noradrenergic) (19, 27, 28). The levels of this enzyme in the adrenal gland are normally regulated by corticosteroids (16). Exposure of PC12 cells (either treated or untreated with NGF) to 10^{-4}–10^{-6} M dexamethasone for 2 weeks, however, does not induce an increase in levels of either this methyltransferase or epinephrine.

The data in Table 1 also indicate that treatment of PC12 cells for 2 weeks with NGF results in little or no change in amine or enzyme levels when expressed on a per cell basis, and a 4- to 6-fold decrease in levels when expressed on a per mg of protein basis.

**DISCUSSION**

The present communication reports the establishment of a single cell clonal line (PC12) of rat pheochromocytoma cells.
which express a number of interesting differentiated properties. Thus far, the PC12 cells have undergone approximately 70 generations since their isolation and have shown no major changes in their cell growth characteristics, morphology, noradrenergic properties, or NGF sensitivity. The homogeneity and near-diploidy of the cell chromosome number suggest that the line will tend to remain genotypically and phenotypically stable in vitro for many generations.

The ability of the PC12 line to respond to NGF is remarkable in view of current knowledge concerning target cells for NGF. Normal adrenal chromaffin cells and their neoplastic counterparts in pheochromocytomas do not possess neuronal processes in vitro, nor do normal adrenal chromaffin cells form processes in response to in vivo treatment with NGF (37). Small, intensely fluorescent extra-adrenal chromaffin-like cells in sympathetic ganglia can extend processes, but such cells neither require nor respond to NGF (36). Sympathetic neurons, on the other hand, do respond to NGF with increased neurite outgrowth (7, 9), but such cells (unlike pheochromocytoma cells) require NGF for survival both in vivo and in vitro (7, 9). The resolution of these apparent inconsistencies may lie in the embryologic origin of sympathetic and chromaffin cells. Both cell types store and secrete catecholamines and both are derived from a common primitive stem cell of neural crest origin (38, 39). It has been suggested that extra-cellular factors may influence the final path of differentiation of such stem cells (30), and there is evidence (40) that NGF plays a role in promoting the neuronal differentiation of cells migrating from neural crest. These considerations suggest that PC12 cells possess the pluripotency of a primitive progenitor which can differentiate along the lines of either chromaffin cells or sympathetic neurons, with NGF promoting their differentiation in a neuronal direction.

The relationship between cessation of cell division and NGF-induced fiber outgrowth in the PC12 line is presently not clear. The comparative time courses of these events suggest that NGF-treated cells undergo at least one round of division prior to neurite outgrowth. Blockade of cell division alone, however, does not appear to be sufficient to promote fiber outgrowth, as evidenced by the lack of morphological differentiation observed in the presence of the mitotic inhibitor cytosine arabinoside or of low serum concentration. Future studies with PC12 cells may be useful in clarifying the relationships between cell division, cell cycle, neuronal differentiation, and neurite outgrowth in the normally developing nervous system.

The influence of NGF on the multiplication and morphology of PC12 cells is also interesting from the standpoint of tumor biology. Human neuroblastomas may occasionally undergo spontaneous maturation into ganglioneuromas, and such changes may be accompanied by improved prognosis (41). Also, human pheochromocytomas may contain cells which are morphologically indistinguishable from ganglion cells (42). The ability of PC12 cells to form tumors makes them amenable to correlative in vitro and in vivo studies of apparently comparable phenomena.

The present data reveal that PC12 cells synthesize and store sizable amounts of the neurotransmitters dopamine and norepinephrine, but not of epinephrine. In these respects, PC12 cells resemble noradrenergic adrenal chromaffin cells and sympathetic neurons. Results to be presented elsewhere (L. A. Greene, G. Rein, and A. S. Tischler, in preparation) show that PC12 cells also possess mechanisms for both the Ca++-dependent exocytotic release and cocaine-sensitive high-affinity uptake of catecholamines. In contrast with sympathetic ganglia and adrenal chromaffin cells (19, 39), however, PC12 cells contain more dopamine than norepinephrine. One possible explanation for this is that the rate of norepinephrine synthesis in PC12 cells is limited by their levels of dopamine $\beta$-hydroxylase. In support of this possibility is the observation that the uncloned pheochromocytoma contains both a higher norepinephrine to dopamine ratio and a higher specific activity of dopamine $\beta$-hydroxylase than do the PC12 cells. Another

<table>
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<tr>
<th>Enzyme</th>
<th>PC12 cells</th>
<th>NGF-treated PC12 cells</th>
<th>Uncloned tumor</th>
<th>Whole rat adrenal glands</th>
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<tr>
<td>Tyrosine hydroxylase</td>
<td>39 ± 5</td>
<td>10 ± 1</td>
<td>24 ± 3</td>
<td>5 ± 1</td>
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<td></td>
<td>(7 ± 1)</td>
<td>(8 ± 1)</td>
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<tr>
<td>Dopa decarboxylase</td>
<td>770 ± 99</td>
<td>130 ± 18</td>
<td>1359 ± 182</td>
<td>458 ± 65</td>
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<td></td>
<td>(135 ± 22)</td>
<td>(104 ± 18)</td>
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<tr>
<td>Dopamine $\beta$-hydroxylase</td>
<td>806 ± 84</td>
<td>161 ± 19</td>
<td>3980 ± 444</td>
<td>268 ± 30</td>
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<tr>
<td></td>
<td>(141 ± 21)</td>
<td>(130 ± 20)</td>
<td></td>
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<tr>
<td>PNMT</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>21 ± 2</td>
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<td>Monoamine oxidase</td>
<td>3.9 ± 0.4</td>
<td>—</td>
<td>9.5 ± 1.2</td>
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<td>Catechol-O-methyltransferase</td>
<td>176 ± 18</td>
<td>—</td>
<td>154 ± 18</td>
<td>156 ± 17</td>
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Catecholamine

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<th>Catecholamine content†</th>
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<tr>
<td>Dopamine</td>
<td>16.6 ± 1.7</td>
<td>4.4 ± 0.4</td>
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<td>(2.9 ± 0.5)</td>
<td>(3.3 ± 0.5)</td>
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<td>Norepinephrine</td>
<td>6.1 ± 6</td>
<td>1.5 ± 0.2</td>
<td>7.5 ± 0.8</td>
<td>4.7 ± 0.5</td>
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<td>(1.0 ± 0.2)</td>
<td>(1.2 ± 0.2)</td>
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<tr>
<td>Epinephrine</td>
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<td>&lt;0.15</td>
<td>&lt;0.15</td>
<td>1.02 ± 0.11</td>
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PC12 cells were grown on collagen-coated 35 mm tissue culture dishes for two weeks with or without the presence of NGF. Assays were performed as described in Materials and Methods with use of either cell homogenates (enzymatic activities) or extracts (catecholamine contents). Protein levels were measured on cell and tissue homogenates; sister cultures were used for cell counts (of PC12 cells) that were made as described in Fig. 2. Values are given as the mean ± SEM; —, not measured; PNMT, phenylethanolamine-N-methyltransferase.

* pmol/min per mg of total cell protein and (pmol/min per 10⁶ cells).
† nmol/mg of total cell protein and (nmol/10⁶ cells).
possible factor could be the make-up of the culture medium. For example, Mains and Patterson (43) found that maximal synthesis of norepinephrine by cultured primary sympathetic neurons required supplementation of the medium with ascorbate at frequent intervals.

NGF treatment of PC12 cells does not appear to affect the level per cell of either noradrenergic enzymes or of the neurotransmitters themselves. The observation that the levels of these substances fell by a factor of 4 to 6 (per mg of total cell protein) indicates that the NGF-treated cells are stimulated to synthesize specific classes of new proteins, many of which are probably related to the production of neuritic processes. It has been reported that treatment of rodents with NGF produces, in the superior cervical sympathetic ganglion, an increase in the specific activities/mg of protein of tyrosine hydroxylase and dopamine β-hydroxylase (44). Our findings suggest either that such effects do not occur in PC12 cells or that higher levels of NGF, longer exposure to NGF, or factors in addition to NGF are required to produce such effects in vitro.

In summary, the PC12 line appears to be a useful model system for the study of numerous problems in neurobiology and neurochemistry. These may include the mechanisms of action of NGF and its role in development and differentiation of neural stem cells; initiation and regulation of neurite outgrowth; and metabolism, storage, uptake and release of catecholamines. PC12 cells may also be useful for studies related to treatment of certain classes of tumors.

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