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C6 glioma cell-conditioned medium induces neurite outgrowth and survival of rat chromaffin cells *in vitro*: Comparison with the effects of nerve growth factor

(cell culture/plasticity/growth factors/catecholamines)

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ABSTRACT **The effects of medium conditioned by rat C6 glioma cells (C6-CM) on the survival, neurite formation, and catecholamine content of adrenal medullary cells in culture were investigated and compared with the effects of nerve growth factor (NGF). Adrenal medullary cells were isolated from 10-day-old rats and the proportions of surviving and neurite-extending cells were determined after 8 days in culture. In the presence of C6-CM virtually all seeded cells survived and 50% developed neuritic processes. In contrast, NGF did not support survival above control levels (30%) and induced neurite formation from approximately one-third of the surviving cells. C6-CM and NGF had no additive effects on neurite outgrowth. C6-CM-mediated fiber outgrowth was not inhibited by physiological concentrations of glucocorticoids which abolished the NGF-induced neurite formation. Both C6-CM and NGF increased the catecholamine content of the cultures and reduced the relative content of epinephrine. However, in view of its substantial effect on cell survival as compared to NGF, C6-CM caused a reduction of the catecholamine content per cell. We conclude that adrenal medullary cells, like other members of the sensory–sympathetic cell lineage of the neural crest, respond to glial-conditioned medium. This response differs both quantitatively and qualitatively from that mediated by NGF.**

Adrenal medullary endocrine cells and sympathetic neurons are closely related to each other with regard to their origin from the neural crest (1) and their capacity to synthesize, store, and release catecholamines and various neuropeptides (2). Although adrenal medullary cells exhibit distinct morphological differences from neurons *in vivo* (3, 4), best characterized by the absence or presence, respectively, of neurites and large chromaffin storage granules, they may adopt morphological features of neurons, such as neurites (5), growth cones (6), small synaptic vesicles (5), tetanus toxin binding sites (7), and neurofilaments (unpublished data), when grown in culture or in transplants (5, 8, 9). A well-defined experimental condition that allows adrenal chromaffin cells to acquire a neuronal morphology is a culture system of dissociated cells, to which nerve growth factor (NGF) has been added (5). With adrenal medullary chromaffin cells taken from early postnatal rats it has been documented that the NGF-induced fiber outgrowth is markedly inhibited by glucocorticoid hormones (5), suggesting that the high glucocorticoid concentrations normally present in the adrenal medulla prevent the formation of neurites by medullary chromaffin cells *in vivo*. Moreover, NGF has been shown to support the survival of neonatal but not 10-day-old rat adrenal chromaffin cells in culture (10).

The requirements of crest-derived neuronal cells for trophic and specifying factors other than NGF that induce neurite outgrowth and transmitter synthesis, regulate the choice of transmitters, and support survival *in vitro* have received increased attention in recent years (for reviews see refs. 11 and 12). Such *in vitro* studies have contributed significantly to our understanding of developmental events in the peripheral autonomic nervous system and of the maintenance of functional competence of these neurons. Similar studies performed with endocrine adrenal medullary cells could help to determine to what extent they differ from other crest-derived cells in their response to factors other than NGF, and to further elucidate their developmental position within the neural crest lineages. An NGF-antibody-resistant activity that supports survival and neurite extension in embryonic chicken sensory and sympathetic neuron cultures and induces neurite extension and choline acetyltransferase activity in PC-12 rat pheochromocytoma cells has been found in media conditioned by glioma cells (C6-CM) and in brain extracts (13–16). We report here that C6-CM applied to cultured adrenal medullary cells from young postnatal rats (*i*) induces neurite outgrowth, (*ii*) shifts the synthesis of catecholaminergic transmitters towards primary amines, and (*iii*) supports survival; however, C6-CM has significant differences as compared to NGF.

MATERIALS AND METHODS

Culture Methods. Adrenal glands were dissected from 10-day-old rats (Hanover-Wistar strain). After the capsule and cortical tissue had been removed the medullae were dissociated into isolated cells as described (5). Isolated adrenal medullary cells were cultured in modified Rose chambers (5), 24-well multiwell plates (Falcon 3047), or modified 35-mm Falcon Petri dishes (17) on glass coverslips coated with a thin film of rat tail collagen at equal densities (50,000 cells per 100 mm^2). The culture medium consisted, unless otherwise stated, of glucose-enriched medium 199 supplemented with 20% (vol/vol) fetal calf serum and gentamycin at 100 μg/ml. The medium was changed 18–24 hr after the cells had been planted to remove dead cells and cell debris and the cultures were supplied with fresh medium containing (*i*) β-NGF at 100 or 1000 ng/ml, (*ii*) C6-CM at various concentrations (see below), (*iii*) β-NGF at 100 ng/ml plus C6-CM, (*iv*) C6-CM plus anti-NGF antibodies inhibiting an equivalent of 50 biological units of NGF, (*v*) C6-CM plus 10 μM disodium dexamethasone 21-phosphate (Merck, Darmstadt, F.R.G.), (*vi*) β-NGF at 100 ng/ml plus 10 μM dexamethasone, (*vii*) 10 μM dexamethasone, and (*viii*) anti-NGF antibodies alone. Experimental and control cultures were incubated for 8 days after addition of factors and the culture medium was changed every 2–3 days.

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Abbreviations: NGF, nerve growth factor; C6-CM, conditioned medium of C6 cells; DEX, dexamethasone.

Cell Counts. Numerical analysis of surviving chromaffin cells and cells displaying neurite outgrowth was carried out at the beginning (18–24 hr after plating) and at the end of the experiments (9 days after plating), using phase-contrast microscopy and catecholamine histofluorescence images. A minimum of two diametric strips was counted at a ×125 magnification in each well. Each culture condition was tested in quadruplicate in each experiment. All experiments were repeated at least twice. Photomicrographs were taken with Agfapan 25 or Kodak Tri-X pan films.

Preparation and Bioassay of NGF. β-NGF from submaxillary glands of male mice was isolated by the method of Varon *et al.* (18). Remaining contaminating proteins were removed by an additional gel filtration step on Sephadex G 75. The biological activity of NGF was determined by the chicken dorsal root ganglion assay (18) and amounted to 100–200 biological units per μg of protein in our preparation.

Preparation and Specificity of Anti-NGF Antibodies. Antisera against the β-subunit of NGF were raised in rabbits. Specificities of the antisera were checked by Ouchterlony double immunodiffusion and by the transblot technique as described (19). The titer was determined on the basis of the inhibitory effect of the antibodies on NGF activity in the ganglion assay.

Preparation of C6-CM. C6 rat glioma cells were cultured in 45% Dulbecco's modified Eagle's medium, 45% Ham's F-12 medium, and 10% fetal calf serum with an antibiotic supplement in 75-cm^2 Falcon flasks. Confluent cultures were

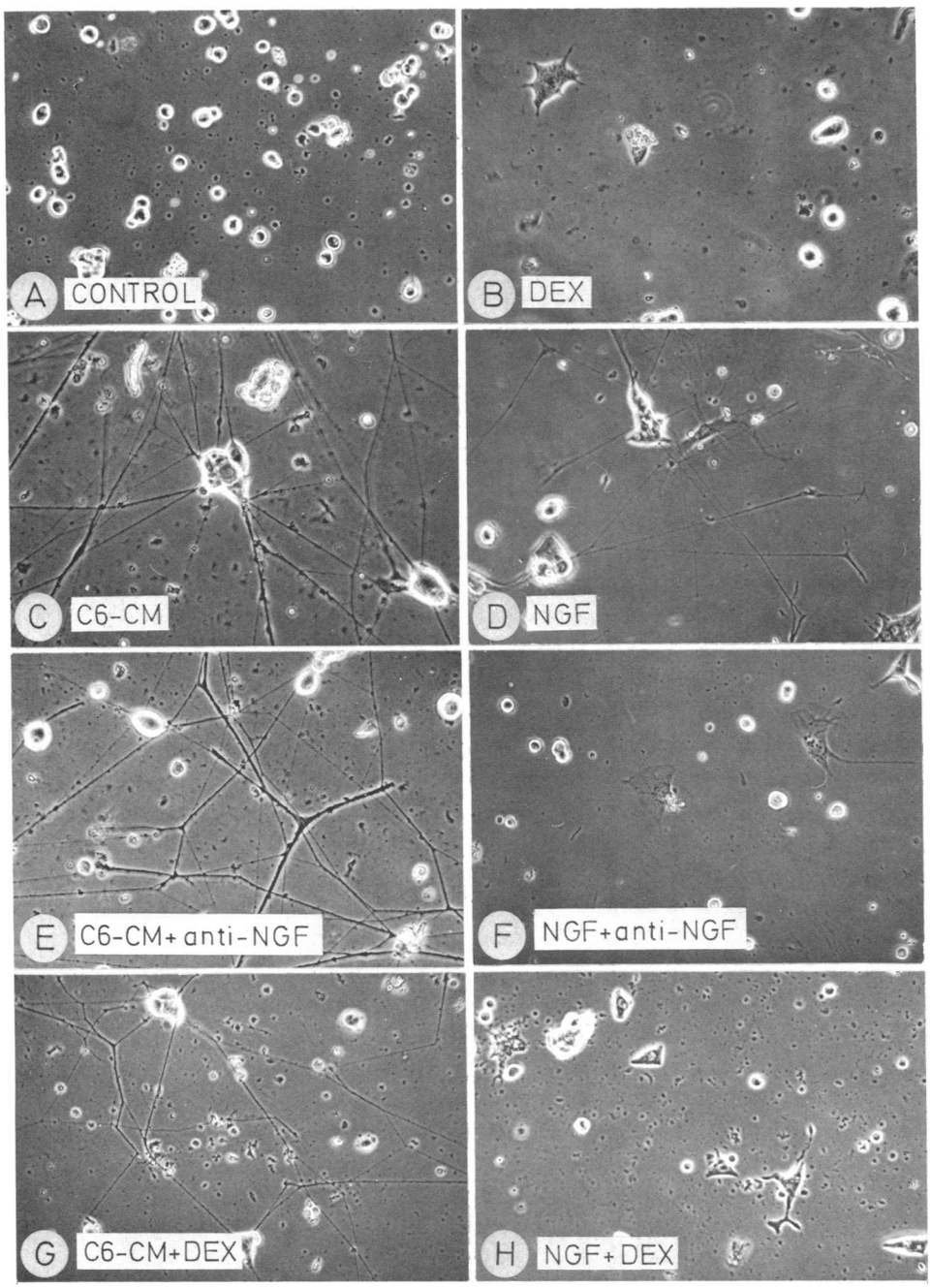

FIG. 1. Phase-contrast micrographs of unfixed adrenal chromaffin cells cultured for 8 days as a control (*A*), in the presence of 10 μM DEX (*B*), with 1-fold concentrated C6-CM (*C*), β-NGF at 100 ng/ml (*D*), C6-CM plus anti-NGF antibodies inhibiting an equivalent of 50 biological units of NGF (*E*), β-NGF at 100 ng/ml plus anti-NGF antibodies inhibiting an equivalent of 50 biological units of NGF (*F*), C6-CM plus 10 μM DEX (*G*), and β-NGF at 100 ng/ml plus 10 μM DEX (*H*). (*A*, *B*, *D*, and *F–H*, ×162; *C* and *E*, ×230).

washed with medium without the addition of fetal calf serum and incubated with serum-free medium, unless otherwise stated, for 4 days. After conditioning, the medium was aspirated from the cells, centrifuged (1000 × *g* for 5 min), filtered through 0.22-μm-pore-diameter Millipore filters, and stored frozen until required, when it was either used directly or diluted with fresh medium (20% fetal calf serum being added). For most experiments C6-CM was concentrated up to 100-fold by ultrafiltration through Amicon filters PM 10 or YM 5, stored frozen, and diluted prior to use.

Catecholamine Histochemistry. After washing in serum-free medium, coverslips were immersed for 3 sec in ice-cold buffered 1% glyoxylic acid and processed according to De La Torre and Surgeon (20).

Electron Microscopy. Cultures were fixed in phosphate-buffered 2.5% (wt/wt) glutaraldehyde, postfixed in 2% aqueous OsO_4, and processed for electron microscopy as described (5).

Quantitative Determinations of Catecholamines. After removal of the culture medium 900 μl of 0.1 M sodium acetate buffer, pH 6.0/1% (wt/vol) Triton X-100/25 mM EDTA and 100 μl of a 250 ng/ml *N*-methyldopamine standard were added to each well. Catecholamines in deproteinized samples were quantified by HPLC with electrochemical detection as described (21).

RESULTS

Control and NGF-Treated Cultures. Isolated adrenomedullary cells cultured in control medium and in the presence of NGF or dexamethasone (DEX, 10 μM) displayed growth characteristics as previously reported (5). There was little, if any, neurite formation by chromaffin cells under control conditions (Fig. 1*A*) and in the presence of DEX (Fig. 1*B*), whereas NGF (100 ng/ml) elicited fiber outgrowth from 30–40% of the chromaffin cell population within 8 days (Fig. 1*D* and Table 1). Higher concentrations of NGF (1000 ng/ml) did not significantly increase the proportions of process-extending cells but caused a more rapid onset of fiber outgrowth. The reported inhibitory effect of DEX (10 μM) on NGF-induced process formation was also confirmed (Fig. 1*H*). NGF did not enhance survival of chromaffin cells beyond control levels (approximately 30% of the initially seeded cells) (Table 1). All chromaffin cells exhibited strong catecholamine-specific histofluorescence.

Response of Chromaffin Cells to C6-CM. C6-CM elicited neurite outgrowth from chromaffin cells (Fig. 1*C*). The morphological pattern of the processes formed was essentially as seen with NGF. However, the proportion of process-extending cells was found to be consistently greater than with NGF (Table 1), and the onset of fiber outgrowth was often observed within 24–48 hr. The relative number of chromaffin cells with processes depended on the concentration of C6-CM. Saturating amounts of C6-CM supplied to the culture medium stimulated 50% and more of the chromaffin cells to grow fibers (Fig. 2). Supplementation of the C6-CM-containing medium with NGF did not further augment fiber outgrowth (Table 1). Antibodies directed against NGF, while able to inhibit the NGF-induced fiber outgrowth from chicken spinal ganglia and young rat chromaffin cells (Fig. 1*F*), did not inhibit the outgrowth induced by C6-CM (Fig. 1*E* and Table 1). Interestingly, in contrast to NGF-stimulated chromaffin cells, DEX (10 μM) did not impair the C6-CM-induced fiber outgrowth (Fig. 1*G* and Table 1).

Table 1. Effects C6-CM, NGF, DEX, and anti-NGF on survival and fiber outgrowth

Experiment	Survival, %	Fiber outgrowth, %
Control	27.5 ± 4.9 (8)	2.5 ± 0.6 (12)
C6-CM	91.6 ± 3.6 (4)	48.1 ± 3.8 (8)
NGF, 100 ng/ml	26.7 ± 6.0 (6)	33.4 ± 3.2 (8)
NGF, 1 μg/ml	ND	34.5 ± 1.6 (2)
C6-CM + DEX, 10 μM	92.9 ± 3.9 (4)	46.5 ± 3.0 (4)
C6-CM + anti-NGF	92.3 ± 3.0 (4)	41.9 ± 6.1 (4)
NGF, 100 ng/ml + DEX, 10 μM	35.7 ± 2.1 (4)	8.2 ± 2.1 (4)
C6-CM + NGF, 100 ng/ml	91.2 ± 3.8 (4)	48.5 ± 3.2 (4)
DEX, 10 μM	35.8 ± 4.5 (4)	4.5 ± 1.8 (4)
Anti-NGF	31.9 ± 6.1 (4)	4.3 ± 2.0 (4)

Effects were measured on adrenal medullary cells from 10-day-old rats after 8 days in culture. Numbers of experiments are given in parentheses. C6-CM was prepared by diluting 10-fold concentrated C6-CM 1:10 with fresh culture medium. ND, not determined.

Catecholamine histochemical and electron microscopic examinations (Fig. 3) of C6-CM-treated chromaffin cells revealed no distinct differences as compared to those challenged with NGF. The ultrastructural features of both NGF- and C6-CM-treated chromaffin cells were very heterogeneous. The cells contained specific storage granules of different sizes and dense bodies in various amounts (Fig. 3). Large dense-cored vesicles varied in diameter from 100 to 250 nm and were frequently smaller in the cell processes than in the perikarya. Small clear and dense-cored vesicles (40–100 nm) were more consistently found in processes than in cell bodies (Fig. 3).

Quantitative analyses of catecholamines (Table 2) by HPLC and electrochemical detection indicated that both C6-CM and NGF caused an increase in the catecholamine content per culture of 165% as compared to controls. Furthermore, the relative portion of epinephrine was significantly reduced from 22% in control cultures to 15% in C6-CM- and NGF-treated cultures. The presence of anti-NGF antibodies did not alter these effects of C6-CM.

In addition to its growth-promoting effect and in apparent contrast to NGF, C6-CM significantly augmented survival of chromaffin cells (Table 1). With saturating concentrations of C6-CM employed, virtually all seeded chromaffin cells present 18–24 hr after plating survived the 8-day culture period. This effect of C6-CM on survival was also dependent on the duration of conditioning (not shown) and was not impaired by administration of anti-NGF antibodies or DEX (Table 1).

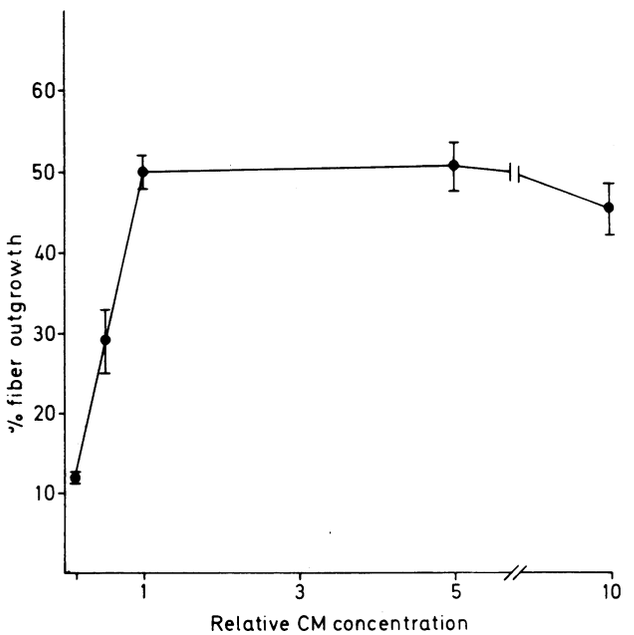

FIG. 2. Concentration dependence of C6-CM-stimulated fiber outgrowth of rat (10-day) chromaffin cells. Relative concentrations were obtained by appropriate dilution of 100-fold concentrated C6-CM with fresh culture medium.

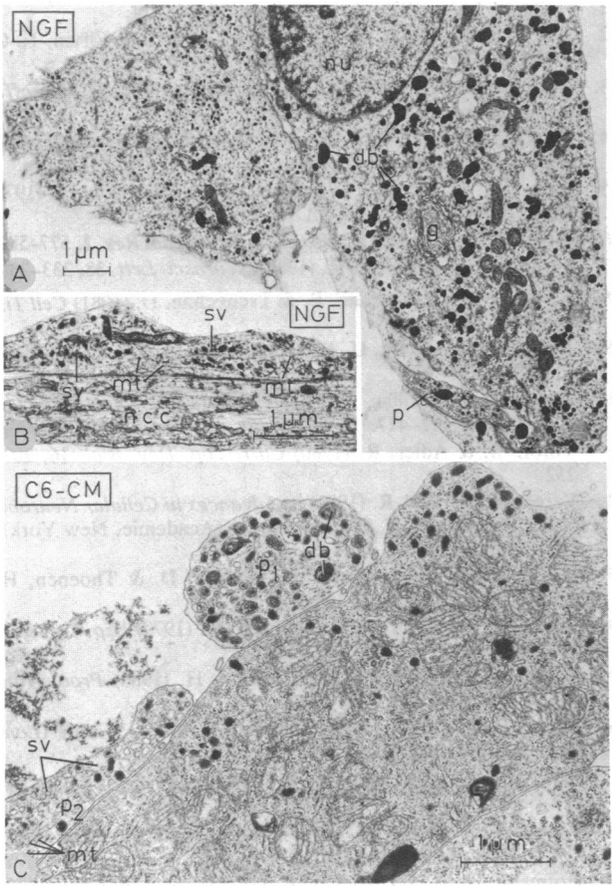

FIG. 3. Electron micrographs of rat adrenal chromaffin cells cultured for 8 days in the presence of β-NGF at 100 ng/ml (*A* and *B*) and 1-fold-concentrated C6-CM (*C*). Note the heterogeneous ultrastructural features of the chromaffin cells and neurite appearance of the processes. nu, Nucleus; db, dense bodies; g, Golgi field; p, processes; sv, small vesicles; mt, microtubules; ncc, non-chromaffin cell.

However, it was found that medium conditioned for 8 days was less effective if undiluted with fresh medium. This effect may be explained either by lack of nutrients or by accumulation of toxic materials.

DISCUSSION

The results presented here show that C6-CM induces fiber outgrowth, increases survival *in vitro*, and changes storage of catecholamines in chromaffin cells dissociated from adrenal glands of 10-day-old rats. C6 glioma cells have been reported to release into their culture medium an activity, other than NGF, that supports neurite extension and survival of embryonic chicken sensory and sympathetic neurons (15, 16) and increases neurite formation and choline acetyltransferase activity in PC 12 cells (14). Although some NGF is contained in C6-CM (15), the effects demonstrated in our study with C6-CM are clearly not attributable to NGF, because addition of antibodies directed against NGF did not inhibit these effects. The quantities of antibodies applied were large enough to block an equivalent of NGF that is higher than the NGF concentration in C6-CM (ref. 22 and unpublished).

The response of young rat chromaffin cells to C6-CM resembles that seen with NGF in several respects. First, the cells extend neurite processes with ultrastructural characteristics identical to those shown in response to NGF (5). Varicosities and growth cones contain large numbers of small clear and dense-cored vesicles similar to those shown by sympathetic neurons *in vivo* and *in vitro* (4). The identity of the small clear vesicles in terms of their putative transmitter remains to be established. Simultaneously, a decrease in the number and size of the large storage granules typical of the endocrine chromaffin cells was noted. These ultrastructural features seen in C6-CM and NGF-stimulated cells and the acquisition of tetanus toxin binding sites (7) by the process-bearing cells clearly point out their neuronal nature.

In agreement with these morphological results, the shift of the relative catecholamine content towards primary amines in the presence of both NGF and C6-CM may be interpreted as an alteration of at least a subpopulation of the cells from an endocrine to a neuron-like cell type.

No major differences were observed with respect to the overall catecholamine increase per culture under both regimens of treatment. Calculation of the catecholamine content per cell, however, revealed completely different effects of NGF and C6-CM. While NGF, which did not support survival, increased the content per cell, a dramatic reduction was observed in the presence of C6-CM. These results indicate that NGF and the factor present in C6-CM have different mechanisms of action, and this distinction is strengthened by the observation that DEX, applied at concentrations that clearly inhibit the NGF-induced fiber outgrowth (5), has no effect on C6-CM-stimulated process formation. In this respect the activitie(s) contained in C6-CM apparently resemble those present in cultured rat adrenal non-chromaffin cells (23) and in cultures of young adult rat adrenal medullae (24), both of which elicit fiber outgrowth of chromaffin cells that is not blocked by DEX. The fact that DEX prevents the NGF- but not the C6-CM-mediated fiber outgrowth also raises the question as to which physiological agent might suppress the effects of C6-CM or related substances *in vivo*, given that they are present in the adrenal medulla *in situ*. A source for glial factors in the adrenal medulla could be the satellite cells, which incompletely ensheath the chromaffin cells (25).

In contrast to NGF, C6-CM permitted the survival of virtually all the chromaffin cells plated. NGF supports the survival of chromaffin cells from newborn rats but becomes less

Table 2. Catecholamine content of cultured chromaffin cells

	Total catecholamines		Dopamine		Norepinephrine		Epinephrine	
Experiment	pmol per culture	fmol per cell	pmol per culture	mol %	pmol per culture	mol %	pmol per culture	mol %
Control (n = 3)	131 ± 7.5	9.5	29 ± 1	22 ± 0.1	73.7 ± 1	56 ± 1.5	28.5 ± 5.4	22 ± 2.1
NGF (n = 3)	220 ± 6†	16.4	19 ± 1†	8.5 ± 1†	170 ± 3*	77 ± 2†	31 ± 2	14 ± 2*
C6-CM (n = 3)	210 ± 24*	4.6	28 ± 1	15 ± 1*	150 ± 23*	69 ± 1†	32 ± 0.5	16 ± 0.6*
C6-CM + anti-NGF (n = 5)	204 ± 20*	4.4	25 ± 3	12 ± 1.4*	142 ± 13*	72 ± 2†	36 ± 4	17 ± 1.2*

Catecholamine content of cultured adrenal medullary cells from 10-day-old rats after 8 days in culture. The total catecholamine content per cell was calculated on the basis of parallel survival experiments (see Table 1), assuming a cell number of 50,000 at 100% survival. Values significantly different from controls are as indicated: *, $P < 0.01$, and †, $P < 0.001$.

effective during the first postnatal week, until, at day 10, equal numbers of chromaffin cells survive with and without NGF (11). The responsiveness of chromaffin cells at day 10 to C6-CM thus mimics the *in vitro* survival characteristics of dissociated chicken sensory (15) and sympathetic neurons (16), which can be supported by C6-CM at later embryonic stages, when they have become less dependent on NGF. It will be interesting to compare the effects of C6-CM and NGF on pre- and postnatal developmental stages of chromaffin cells earlier than day 10. Chromaffin cells from 30-day-old rats have recently been shown to form processes when treated with C6-CM but are no longer responsive to NGF, whilst chromaffin cells from adult bovine adrenal glands respond neither to C6-CM nor to NGF in terms of fiber outgrowth (unpublished results). Taken together, these facts suggest that the responsiveness of chromaffin cells *in vitro* towards neurite outgrowth-promoting factors declines with age.

C6-CM was consistently more potent than NGF with respect to eliciting fiber outgrowth from 10-day-old rat chromaffin cells. This does not necessarily indicate a greater responsiveness of the cells, but it may reflect the larger proportion of surviving cells as compared to NGF. NGF and C6-CM applied together had neither additive nor potentiating effects on the chromaffin cells' capacity to extend neurites. This suggests that the NGF-responsive cells are contained within the C6-CM-responsive cell subpopulation. However, their effects on chromaffin cell survival allow a clear distinction to be made between NGF and the activity contained in C6-CM. Our results allow the recognition of at least two subpopulations of chromaffin cells with distinct neuronal or endocrine phenotypes. Attempts to further characterize these subpopulations by any other means except tetanus toxin binding have not been successful so far. [Met]enkephalin immunoreactivity (unpublished observations) may be seen in cells both with and without neuronal processes. The fact, however, that the addition of a factor from C6-CM to the culture medium keeps virtually all the medullary cells surviving opens the possibility to characterize the subpopulations of cells that obviously are present in primary cultures of adrenal chromaffin cells.

In conclusion, juvenile rat adrenal chromaffin cells kept in tissue culture respond to C6-CM by fiber outgrowth and enhanced survival. The activity (activities) mediating these effects are distinguishable from NGF, since they cannot be blocked by anti-NGF antibodies and DEX, and since they support survival at a developmental stage at which NGF is no longer effective.

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