ROLE OF NERVE GROWTH FACTOR IN THE DEVELOPMENT OF RAT SYMPATHETIC NEURONS IN VITRO

III. Effect on Acetylcholine Production

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ABSTRACT

The effect of nerve growth factor (NGF) on the development of cholinergic sympathetic neurons was studied in cultures grown either on monolayers of dissociated rat heart cells or in medium conditioned by them. In the presence of rat heart cells the absolute requirement of neurons for exogenous NGF was partially spared. The ability of heart cells to support neuronal survival was due at least in part to production of a diffusible NGF-like substance into the medium. Although some neurons survived on the heart cell monolayer without added NGF, increased levels of exogenous NGF increased neuronal survival until saturation was achieved at 0.5 μg/ml 7S NGF. The ability of neurons to produce acetylcholine (ACh) from choline was also dependent on the level of exogenous NGF. In mixed neuron-heart cell cultures, NGF increased both ACh and catecholamine (CA) production per neuron to the same extent; saturation occurred at 1 μg/ml 7S NGF. As cholinergic neurons developed in culture, they became less dependent on NGF for survival and ACh production, but even in older cultures ~40% of the neurons died when NGF was withdrawn. Thus, NGF is as necessary for survival, growth, and differentiation of sympathetic neurons when the neurons express cholinergic functions as when the neurons express adrenergic functions (4, 5).

KEY WORDS nerve growth factor · sympathetic neurons · cell culture · acetylcholine production

As described in the initial paper of this series (4), nerve growth factor (NGF) causes dose-dependent increases in survival, growth, and differentiation of catecholamine (CA) production in cultures of dissociated rat sympathetic neurons. Although most of the principal cells in the adult rat superior cervical ganglion (SCG) are adrenergic, Yamauchi et al. (25) obtained histochemical evidence suggesting, in analogy with cat sympathetic ganglia (23), that ~5% of the neurons are cholinergic. Does NGF also influence the development of sympathetic neurons which utilize acetylcholine (ACh) as transmitter, or is it specific for adrenergic function? It was possible to examine this question in culture, since we have previously demonstrated that sympathetic neurons from the rat SCG can be induced to produce substantial amounts of ACh when grown in vitro in the presence of certain types of non-neuronal cells (20) or in medium conditioned by them (CM) (21, 22). Under these conditions, the neurons also secrete ACh at functional cholinergic syn-

apses which they make with each other (8, 10, 18, 19), with skeletal muscle (16), and with cardiac myocytes (6). We present evidence that NGF affects the development of these cholinergic sympathetic neurons. A preliminary report of some of these findings has appeared (3).

MATERIALS AND METHODS

Many of the methods used in this study were described in the preceding two papers in this series (4, 5) and in a previous report (21). Briefly, mechanically dissociated SCG cells from neonatal rats were grown in L15-CO_2 medium under the following conditions: (*a*) on a monolayer of dissociated rat heart cells and irradiated with ^{60}Co 2 days after neurons were plated to block further division of heart cells and ganglionic non-neuronal cells ("mixed cultures"), (*b*) by themselves and treated with 10^{-5} M cytosine arabinoside from days 2–4, 6–8, and 15–17 to kill proliferating ganglionic non-neuronal cells, (*c*) by themselves as in (*b*) but treated with CM obtained from flasks of confluent non-neuronal cells. Some cultures were grown in L15-air medium which also suppresses survival and proliferation of ganglionic non-neuronal cells (15).

Both 7S and low molecular weight (LMW) NGF were prepared from submaxillary glands of male mice as previously described (4). Flasks and monolayer cultures of primary heart cells were prepared as described by Patterson and Chun (21). CM was prepared by incubating L15-CO_2 growth medium, with or without added NGF, in flasks of confluent primary rat heart cells for 2 days. The CM was pooled and frozen in aliquots. The neuronal cultures were provided with CM diluted with fresh medium to the desired concentration, and the medium was changed every 2 days. After growth for 3–4 wk in appropriate concentrations of NGF, the cultures were assayed for number of neuronal somas, by scanning the entire well, and for their ability to synthesize and accumulate [^{3}H]CA and [^{3}H]ACh during 4-h incubations with [^{3}H]tyrosine and [^{3}H]choline (21).

In some experiments, L15-CO_2 growth medium containing 5% heat-inactivated rat serum (HIRS) and no exogenous NGF was used to produce CM on C6 rat glioma cells. Neuronal cultures were then grown in glioma CM diluted with fresh medium containing 5% HIRS to which 5 μg/ml 7S NGF had been added. At various ages, sister cultures were rinsed five times with growth medium lacking Methocel (Dow Corning Corp., Midland, Mich.) and NGF; the cultures were then provided with 62% glioma CM containing one of the following: (*a*) 5 μg/ml 7S NGF (control), (*b*) 25 μg/ml anti-NGF rabbit IgG (no added NGF), or (*c*) 25 μg/ml anti-NGF rabbit IgG and 5 μg/ml 7S NGF. After 10 days under these conditions, the cultures were assayed as described above.

The anti-NGF rabbit IgG was a generous gift of Dr. Richard A. Murphy (Department of Anatomy, Harvard Medical School), who determined that 13 μg anti-NGF blocked the fiber outgrowth from chick sensory ganglia elicited by 10 ng 2.5S NGF.

RESULTS

Effect of NGF on Neuronal Survival in the Presence of Heart Cells

Immature, dissociated rat sympathetic neurons cultured in the virtual absence of other cell types require NGF for survival (4). However, when these neurons were co-cultured with cells from rat heart, some survived even in the absence of added NGF as shown in Fig. 1; survival was greater on high density heart monolayers than on low density monolayers (Fig. 1). The survival in the absence of exogenous NGF was due at least in part to production of an NGF-like substance by heart cells. As can be seen in Table I, medium conditioned by heart cells in the absence of NGF maintained neurons in cultures virtually free of ganglionic non-neuronal cells. This property of CM was blocked by anti-NGF (Table I).

Neuronal survival on the heart monolayers increased when the NGF concentration increased; saturation was achieved at 0.5 μg/ml 7S NGF (Fig. 1). Survival remained constant at concentrations up to 10 μg/ml but at 50 μg/ml a cell loss of ~20% was observed. (The loss in Fig. 1 is not statistically significant but several

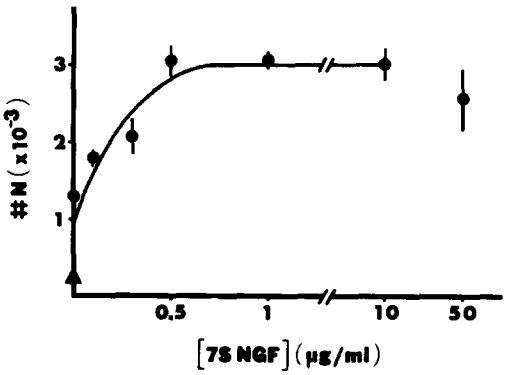

FIGURE 1 Neurons (*N*) were grown on high density monolayers of heart cells (●) (50,000 cells plated per well) or low density monolayers (▲) (5,000 cells plated per well) in the indicated concentrations of 7S NGF, and after 27 days neuronal somas were counted. In this and all subsequent figures, the results are expressed as the mean of at least three sister cultures ± SEM.

TABLE I

Effect of Heart Cell CM on Neuronal Survival and Transmitter Production

	No. neurons/culture	Accumulated ACh/neuron	Accumulated CA/neuron
		fmol/4 h	*fmol/4 h*
25% CM	260 ± 58	0.4 ± 0.1	1.7 ± 0.1
25% CM + 25 μg/ml anti-NGF rabbit IgG*	0	–	–
25% CM + 25 μg/ml anti-NGF rabbit IgG + 5 μg/ml 7S NGF‡	2,245 ± 176	71.1 ± 5.8	6.7 ± 0.7

Neuronal cultures, virtually free of other cell types, were grown for 16 days with CM produced in the absence of added NGF in flasks of confluent rat heart cells.

* Neurons were provided with CM incubated with anti-NGF for 30 min at 37°C.

‡ Neurons were provided with CM incubated with anti-NGF for 30 min at 37°C after which 5 μg/ml 7S NGF was added.

other experiments indicate this decrease was real.) Similar results were obtained with LMW NGF (data not shown).

Effect of NGF on Transmitter Production in Sympathetic Neurons

To determine whether NGF promotes ACh production, as it does CA production (4), neurons were grown on heart cell monolayers in various levels of 7S NGF. The ability of cultures to accumulate synthesized [^{3}H]ACh (accumulated ACh) and [^{3}H]CA (accumulated CA) during a 4-h incubation with [^{3}H]choline and [^{3}H]tyrosine was measured; these rates reflected the sum of all the processes involved in ACh or CA synthesis, storage, and break-down. As shown in Fig. 2, NGF increased both ACh and CA production per neuron, in parallel, in the neuron-heart cultures. Saturation was reached for both at 1 μg/ml 7S NGF, and no inhibition of transmitter production was observed at higher NGF concentrations. Similar results were obtained with LMW NGF (data not shown). Thus, not only did cultures produce ACh under the influence of NGF, but NGF stimulated production of ACh and CA to the same extent. This similarity is further emphasized by examining the relative rates of synthesis and accumulation of both transmitters, the ratio of ACh to CA, as a function of NGF concentration. As can be seen in Fig. 3, whether the neurons were grown on heart cells (Fig. 3 A) or in the virtual absence of other cell types ("neuron-alone cultures," Fig. 3 B), ACh/CA remained constant over a large NGF concentration range. The neuron-alone cultures grown in concentrations of 7S NGF as high as 50 μg/ml, but without CM, produced very little ACh from [^{3}H]choline even in the presence of eserine, an acetylcholinesterase inhibitor. At the highest level of NGF used (50 μg/ml), the cultures produced only 0.25 fmol ACh per neuron compared to 8.9 fmol CA per neuron during an 8-h incubation. Regardless of NGF concentration, these cultures synthesized and accumulated 40-fold more CA than ACh. In contrast, neurons co-cultured with heart cells (Fig. 3 A) were induced to make large amounts of ACh (21), and ACh/CA was much larger than in the neuron-alone cultures. In both cases, however, the ACh-to-CA ratio remained constant over the entire range of NGF concentrations. These data demonstrate that NGF is not specific for adrenergic function but stimulates production of both ACh

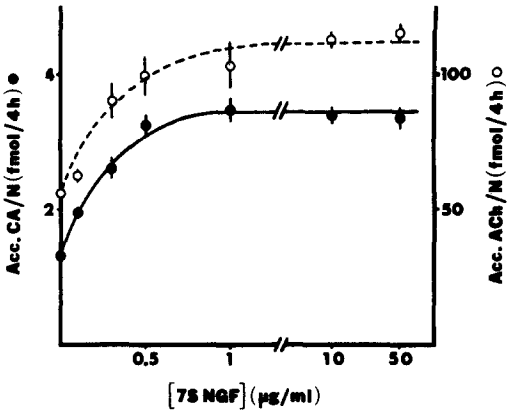

FIGURE 2 Neurons were grown on dense monolayers of rat heart cells in the indicated concentrations of 7S NGF. After 27 days they were incubated for 4 h with [^{3}H]tyrosine and [^{3}H]choline, and the radioactivity accumulated in the cells as CA and ACh was determined (21) and expressed per neuron (N).

and CA. The fact that NGF promotes both kinds of development suggests that NGF is not the diffusible "factor" produced by heart and other cell types which induces cholinergic function (21).

The difference between the cholinergic factor and NGF is emphasized by another experiment. As shown in Table II, medium conditioned by heart cells in the absence of exogenously added NGF still induced the ACh effect; this suggests that the non-neuronal cells did not modify the exogenously added NGF to produce the cholinergic signal. Furthermore, when CM was first incubated with enough anti-NGF to bind the NGF-like substance produced by heart cells and then with excess 7S NGF to allow the neurons to survive, it still induced ACh production (Table I). However, the ratio of ACh to CA was lower for cultures grown in 25% CM without exogenous NGF than in those to which excess 7S NGF had been added. This may have been due to a heterogeneity in the cell population as suggested previously (4, 5); the few neurons able to survive in the presence of the NGF-like substance produced by heart cells may have been less susceptible to the effects of the cholinergic factor.

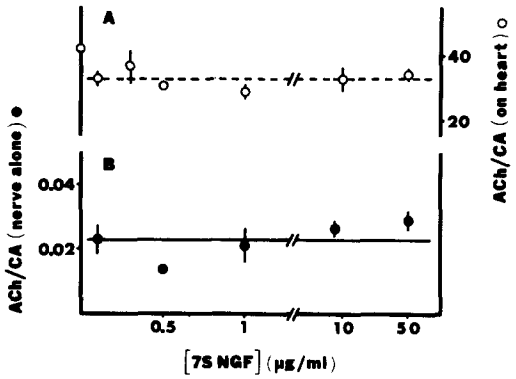

FIGURE 3 The relative production of transmitters by neuron alone and mixed cultures was determined. In (A) neurons were grown on a monolayer of heart cells in the indicated NGF concentrations. After 27 days they were assayed for transmitter production in a 4-h incubation in the absence of eserine. In (B) neurons were grown in the virtual absence of other cell types and without CM in the indicated NGF concentrations. After 23 days they were assayed for transmitter production in an 8-h incubation in the presence of 15 μg/ml eserine sulfate.

Changes in NGF Dependence with Age

In cultures grown in 62% CM, a minimum of 45% of the neurons form functional cholinergic synapses with each other (14). These cholinergic sympathetic neurons, like adrenergic ones (5) exhibited a strong dependence on NGF at early times and became less dependent with age in culture. After 10 days in CM containing NGF, sister cultures were given CM containing anti-NGF and no exogenous NGF. 10 days later, only 10% of the neurons had survived compared to control cultures, as shown in Fig. 4 A. However, when NGF was removed from older cultures, 60% of the neurons remained 10 days later (Fig. 4 A). Similarly, the ability of neurons to produce ACh was also more susceptible to NGF removal in younger cultures, as can be seen in Fig. 4 B. 10 days after NGF withdrawal, the youngest cultures tested produced only 14% of the ACh found in controls. On the other hand, if NGF were withdrawn for 10 days from older cultures, whose capacity for ACh production had matured (P. H. Patterson and L. L. Y. Chun, manuscript in preparation), the cultures synthesized and accumulated 60% of control ACh (Fig. 4 B). Thus, both neuronal survival and ACh production became less dependent on exogenous NGF as the neurons matured.

DISCUSSION

In the presence of heart cells from neonatal rats, the absolute requirement of sympathetic neurons

TABLE II

Effect of CM Prepared in the Absence of Exogenous NGF

NGF added to heart flask	NGF added to neurons	No. neurons/culture	Accumulated ACh/neuron	Accumulated CA/neuron
			fmol/4 h	*fmol/4 h*
+	+	2,330 ± 70	10.6 ± 0.4	97.5 ± 1.1
−	+	2,156 ± 290	11.9 ± 0.7	102.6 ± 5.2

Neuronal cultures were grown for 30 days in 62% CM in a final concentration of 1 μg/ml 7S NGF.

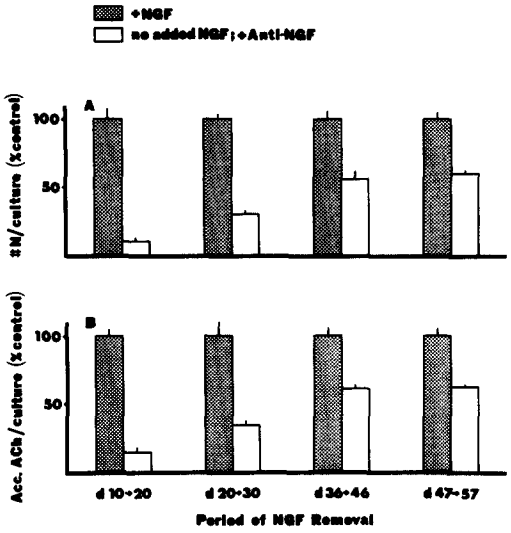

FIGURE 4 The susceptibility of neurons (N) of various ages grown in 62% glioma CM to NGF removal was determined by withdrawing NGF for 10-day intervals between the ages indicated. Cell number (A) and ability to synthesize and accumulate ACh from [^{3}H]choline during a 4-h incubation (B) were assayed at the end of each period of removal.

for exogeneously added NGF was partially spared. The magnitude of neuronal survival depended on the density of non-neuronal cells present as was first observed for dorsal root ganglion cells by Burnham et al. (2). These results are consistent with reports that certain non-neuronal cells secrete an NGF-like substance (17, 24, 26). Since anti-NGF blocked the ability of CM to permit neuronal survival, at least part of the effect of heart cells was to produce an NGF-like substance.

It was possible that increased levels of NGF itself might be the signal responsible for causing sympathetic neurons to become cholinergic, since medium conditioned by rat fibroblasts can induce ACh production in sympathetic neurons (21) and fibroblasts can secrete NGF (26). Several lines of evidence suggest that this is not the case. First, CM incubated with anti-NGF and to which excess 7S NGF was subsequently added, still induced ACh synthesis; this suggests that the NGF-like substance in heart cell CM is probably not the cholinergic signal. However, it is conceivable that the excess 7S NGF displaced the NGF-like substance bound to the antibody. Second, medium conditioned in the absence of NGF still induced ACh production which suggests that the non-neuronal cells do not modify the exogeneously added NGF to produce the cholinergic signal. Finally, neuron-alone cultures grown in concentrations of mouse submaxillary 7S NGF as high as 50 μg/ml, but without CM, produced very little ACh.

Our results indicate that NGF is not specific for either adrenergic or cholinergic function but promotes the differentiation of sympathetic neurons independent of the transmitter they produce. (*a*) Both 7S and LMW NGF stimulated the synthesis and accumulation of ACh and CA to the same extent in a dose-dependent fashion (see also references 4 and 7). (*b*) The cholinergic and adrenergic cultures responded similarly to NGF withdrawal (cf. reference 5). Young sympathetic neurons cultured in the virtual absence of other cell types, are more dependent on NGF for survival than older ones, as was first demonstrated by Lazarus et al. (12, see also reference 1). In the present experiments, NGF removal also affected both ACh and CA production. As the capacity for transmitter production matured, both cholinergic and adrenergic neurons became less dependent on exogenously added NGF. The NGF dependence of cholinergic sympathetic neurons was also suggested by Klingman's (9) observation that, in immunosympathetomized rat SCG, there was no preferential survival of neurons with marked intracellular acetylcholinesterase activity, which was taken to reflect cholinergic function. Therefore, neurons which survive NGF withdrawal are not selected on the basis of which transmitter they produce. In addition, dorsal root ganglion cells, which are not adrenergic, also require NGF during a limited period of embryonic life (13).

Thus, with respect to neurotransmitter synthesis, NGF is permissive rather than instructive; it is necessary for survival and stimulates growth and differentiation but does not instruct the neurons which transmitter to produce. Therefore, NGF is a qualitatively different signal than that in CM, which does not greatly increase neuronal survival or growth but does influence the choice of transmitter produced (21) and type of synapse formed (11) by sympathetic neurons.

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