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Nerve growth factor-induced transformation of immature chromaffin cells *in vivo* into sympathetic neurons: Effect of antiserum to nerve growth factor

(primitive sympathetic cells/pheochromoblasts/adrenal medulla)

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Contributed by Rita Levi-Montalcini, November 29, 1978

ABSTRACT **Pre- and postnatal injections of nerve growth factor, initiated with one dose on day 17 of gestation and continued after birth with daily subcutaneous administration until day 10 of life, produce massive transformation of chromaffin in sympathetic nerve cells in the rat adrenal medulla. Large sympathetic ganglia, absent in controls, adhere to the medial external surface of the gland. Nerve fibers produced by the transformed chromaffin cells invade the inner and outer cortical zones of the organ, producing cell depletion and substantial alteration of the structure of the cortical layers. When the growth factor treatment is initiated after birth, only a partial replacement of chromaffin with nerve cells takes place. The treatment is ineffective after the second postnatal week. Injections of a specific antiserum to nerve growth factor in 17-day-old rat fetuses, which were continued after birth, produce progressive and massive destruction of chromaffin cell precursors and of immature chromaffin cells in the adrenal medullary gland.**

Sympathetic neurons and adrenal and extra-adrenal chromaffin cells share a common origin from stem cells originating in the neuroectoderm. These two cell types synthesize, store, and release catecholamines, but their structures and functions are markedly different. Studies performed in our laboratory showed that chromaffin cells, at variance with sympathetic cells, do not undergo any morphological change in young and adult rodents injected with nerve growth factor (NGF) (1, 2). The finding of an unusually large number of sympathetic nerve cells among chromaffin cells in the adrenal medulla of rats injected with NGF from the day of birth to the third postnatal week raised the question of the origin of these cells. The results of studies to be reported here give unequivocal evidence for the transformation of chromaffin cell precursors and of immature chromaffin cells into sympathetic cells. Thus we show that NGF can divert chromaffin cells that are not fully differentiated and their precursors into nerve cells indistinguishable by morphological and ultrastructural criteria from sympathetic neurons.

Preliminary results of these studies were presented at the 4th European Molecular Biology Organization Annual Symposium on Molecular Neurobiology, Heidelberg, Germany, May 1978.

MATERIALS AND METHODS

Animals. Forty pregnant rats (Sprague–Dawley) raised in our animal quarters were divided into two groups: 26 rats were operated on as indicated below on day 17 of pregnancy (determined on the basis of the observation of the vaginal plug) and 14 rats were housed in single cages. Upon delivery, half of the pups of 10 litters were injected with 10 μg of NGF per g body weight, half of the pups of 4 litters with the immunoglobulin fraction of a specific antiserum to NGF (AS-NGF), and the remaining pups of the 14 litters with vehicle solution. Adult albino mice were purchased from Nossan (Milan, Italy), and the submaxillary glands were removed and used for preparation of NGF.

Materials. NGF was prepared from mouse submaxillary glands by the method of Bocchini and Angeletti (3). The lyophilized protein was dissolved in physiological solution and injected subcutaneously into fetuses or into newborn and infant rats. AS-NGF was prepared as reported (4), and the IgG fraction was separated from the serum by precipitation with ammonium sulfate at 50% saturation.

Surgical Procedures. Pregnant rats were anesthetized with ether and fastened to the operating table. When the anesthesia was complete, a midline abdominal incision about 5 cm long was made and the skin was retracted on either side with hemostatic tweezers and pulled laterally. An aperture through the muscular wall was produced with a sterile bistoury and the right and left parts of the uterus were exteriorized. Each fetus was injected by means of a 30-gauge needle inserted through the intact tube and the skin of the dorsal side of the body. Eighty fetuses were injected with 40 μg of NGF dissolved in 20 μg of physiological solution (group a), 27 with 20 μg of AS-NGF (group b), and 40 with vehicle solution (group c). In all cases care was taken to minimize loss of allantoid and amniotic fluid. After the treatment, the muscle wound was closed with several stitches by using a fine, curved, surgical needle. One litter of group a and one of groups b and c were killed 48 hr after the intrauterine injection; in the remaining cases the operated rats delivered at the end of the gestation period living and apparently normal litters. All pups received from the day of birth NGF, AS-NGF, or vehicle solution according to the protocols of the fetal treatment. The injections were repeated daily until the rats were killed.

Light Microscopy. At daily intervals after birth, pups injected with NGF, AS-NGF, or vehicle solution were killed by decapitation. The superior cervical ganglia and the adrenal glands were removed, fixed in alcoholic Bouin, embedded in Paraplast, sectioned at 8 μm, and stained with toluidine blue or hematoxylin/eosin. Some adrenal glands were stained by the silver technique.

Fluorescence Microscopy. Rats were injected with nialamide, and after 5–6 hr they were killed. The adrenal glands were rapidly frozen in isopentane, cooled in liquid nitrogen, and freeze-dried for 5 days at −40°C in an Edwards high-vacuum dryer. The tissues were subsequently incubated for 1

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Abbreviations: NGF, nerve growth factor; AS-NGF, antiserum to NGF.

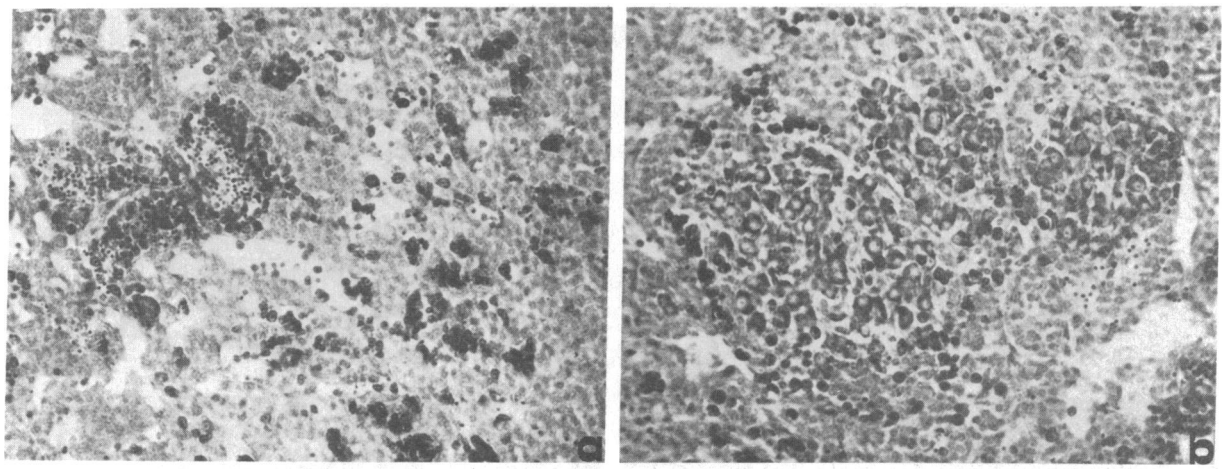

FIG. 1. (*a*) Small clusters of primitive sympathetic nerve cells in a control 19-day-old rat fetus. (*b*) Large aggregate of neuroblasts in the adrenal medulla of a NGF-treated 19-day-old fetus. (×170.)

hr at 80°C with paraformaldehyde vapor calibrated at 70% relative humidity, embedded in Paraplast, sectioned at 7 μm, and examined with a Zeiss fluorescence microscope (5).

Electron Microscopy. As soon as the fetuses or infant rats were killed, the adrenal glands were immersed for 2 hr in glutaraldehyde/paraformaldehyde (5) and postfixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) containing 5% sucrose. The tissues were then dehydrated through graded alcohol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

RESULTS

Light and electron microscopic studies

Control Fetuses and Neonatal Rats. Precursor cells designated as primitive sympathetic cells (6) migrate from the neuroectoderm between the end of the second and the third week of fetal life and settle in small niches among the loosely assembled cordons of the adrenal gland. They colonize in preference the core of the gland, which becomes the adrenal medulla, in close proximity to the endothelial wall of the expanding vascular network. Other cell islets of the same origin and morphological characteristics (e.g., small size, intensely basophilic nucleus, and scanty cytoplasm) adhere to the medial external surface of the gland or form small scattered groups in the gland cortical zones. In the last 2 days of gestation most of the latter groups become surrounded by macrophages and lymphocytes and undergo reabsorption, while the centrally lodged cells increase in number by addition of new units and by proliferative activity. Primitive sympathetic cells are transformed into chromaffin cells through an intermediate stage referred to as pheochromoblast (6, 7). These cells differ from their precursors in their larger nuclei with less dense chromatin and more abundant and slightly basophilic cytoplasm. At birth, the majority of cells have attained this stage and some already show a number of membrane-bound eosinophilic granules characteristic of immature chromaffin cells. Clusters positioned in close proximity of the gland on its medial surface also consist of a heterogeneous cell population towards the end of the gestation period. These small cell aggregates decrease progressively in size and are not detectable anymore at the end of the first postnatal week. After birth, the adrenal medulla increases in volume through the progressive expansion of the vascular network and enlargement of individual cells. Some days later, distinct boundaries become apparent between the inner cortical layer and the adrenal medulla. A few ganglionic cells are irregularly scattered among immature chromaffin cells, pheochromoblasts, and a few residual primitive sympathetic cells.

Electron microscopic studies centered on cells that already show signs of differentiation: pheochromoblasts and immature chromaffin cells. The two cell types are similar in the ultrastructure of the round or ovoidal nucleus, which contains scattered chromatin granules, in the moderate number of mitochondria, and in the rather poorly developed Golgi complex and endoplasmic reticulum. The perikaryon of immature chromaffin cells differs from that of their immediate precursors in the presence of membrane-bound osmiophilic granules with an electron core varying in size from 50 to 200 μm.

Fetuses and Neonatal Rats Injected with NGF. Injections of NGF into 17-day-old fetuses dramatically alter the migratory and differentiative processes of chromaffin cell precursors. The cell clusters adhering to the medial surface of the gland appear markedly larger than in controls. Forty-eight hours after the NGF injections, they exhibit unmistakable marks of immature nerve cells in the prominent nucleus with one or two nucleoli, in their ovoidal profile, and in the slightly basophilic cytoplasm which is markedly broader than in sympathetic primitive cells and in pheochromoblasts. Likewise, cells that have migrated inside the gland give rise to larger aggregates than controls, and individual cells are indistinguishable by light microscopy from neuroblasts of sympathetic ganglia at the same developmental stage. Fig. 1 compares clusters of cells in a control and in a NGF-injected 19-day-old fetus. NGF injections, resumed immediately after birth and repeated daily until day 10, bring to completion the differentiative processes in neuroblasts outside and inside the adrenal gland. At 4 days most of the cells have produced a thin elongated process. Neurites emerging from the large aggregates in the central core of the gland assemble in fascicles that leave the adrenal medulla and perpendicularly cut the inner, medial, and outer cortical zones. Here they reassemble and run in a circular fashion beneath the gland capsule. Fig. 2 *a* and *b* reproduces at low magnification the largest sections of a control and an experimental adrenal gland; Fig. 2 *c* and *d* shows comparable sections of the adrenal medulla of the same glands in two 10-day-old pups. The pre- and postnatal NGF treatment results in a total transformation of the whole gland. The extra-adrenal ganglionic agglomerate, absent in the control (Fig. 2*a*) reaches, in the experimental tissue (Fig.

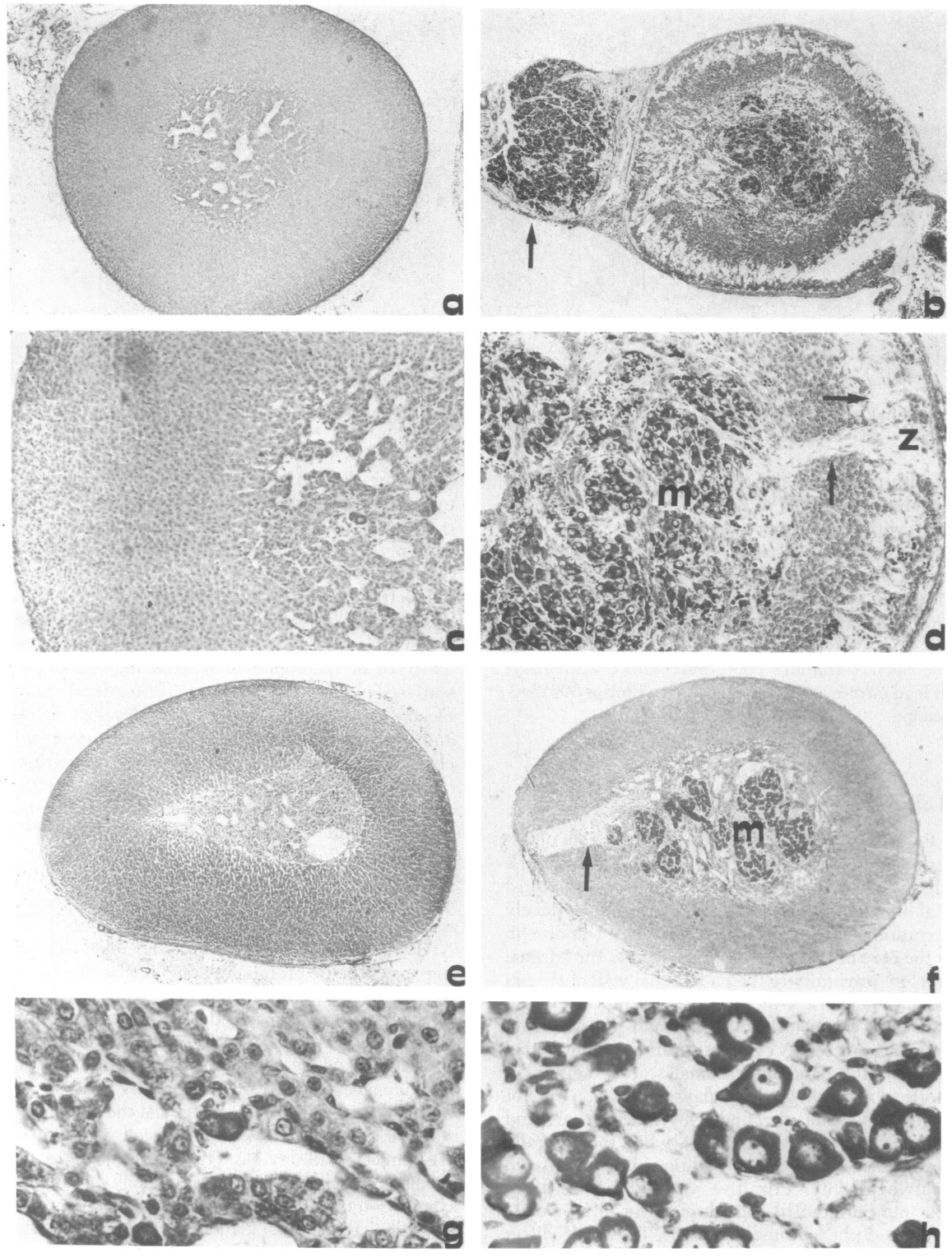

FIG. 2. (*a* and *b*) Largest cross sections of adrenal medulla of two 10-day-old rats injected pre- and postnatally with vehicle solution (*a*) or NGF (*b*). Arrow in *b* points to a ganglionic complex. (×42.) (*c* and *d*) Details at higher magnification of control (*c*) and medullary area (*d*) of adrenal glands shown in *a* and *b*. Arrows in *d* point to fiber bundles originating from transformed nerve cells in adrenal medulla (m) and to glomerular zone (z) invaded by nerve fibers. (×130.) (*e* and *f*) Largest cross sections of the adrenal glands of two 21-day-old rats injected since birth with vehicle solution (*e*) or NGF (*f*). Arrow in *f* points to large fiber bundles produced by transformed nerve cells in the adrenal medulla (m). (×24.) (*a* and *h*) Chromaffin (*g*) and nerve cells (*h*) of adrenal medulla reproduced, respectively, in *e* and *f*. (×460.)

2*b*), a volume comparable to that of the adrenal medulla. Fibers emerging from this *de novo* formed ganglion join those that originate in the adrenal medulla. The marked reduction in width of the cortical layers is due to invasion of the inner zone by nerve fibers and neurons that have replaced the chromaffin cells in the adrenal medulla and by the accumulation of axon bundles in the outer cortical zone greatly depleted of cells. Studies in silver-stained preparations showed that the fibers end

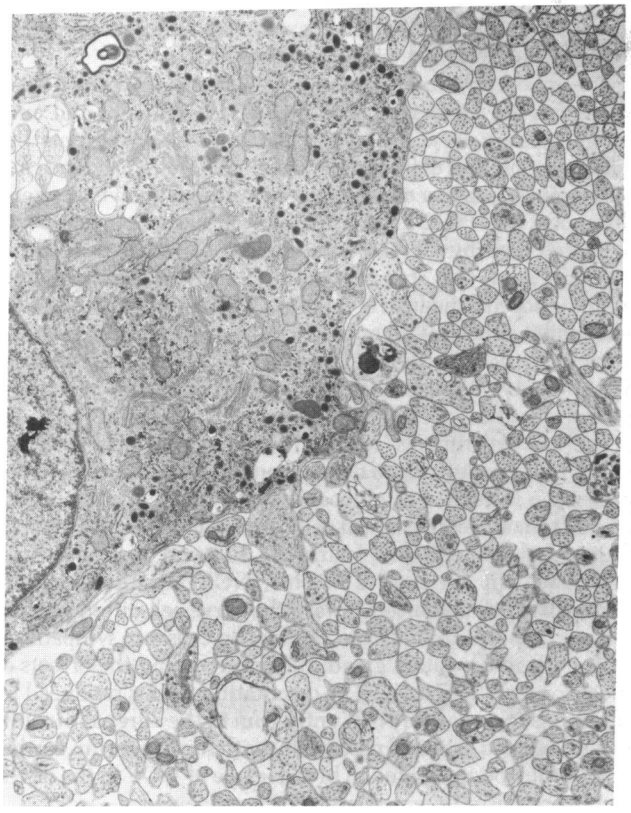

FIG. 3. Electron micrograph of part of a cell exhibiting nerve and glandular features in the adrenal medulla of an 8-day-old rat injected pre- and postnatally with NGF. (×6000.)

freely without establishing connections with the loosely packed, sparse cortical cells. In the adrenal medulla (Fig. 2*d*) nerve cells and fibers obliterate the vascular network; their distribution markedly differs from that of chromaffin cells and resembles that of nerve cells in sympathetic ganglia.

The postnatal NGF treatment differs from that initiated in fetuses in the partial, rather than massive, transformation of chromaffin cell precursors and immature chromaffin cells in nerve cells. Compact islands of nerve cells are intermingled with small chromaffin cells assembled around and among venous sinuses and small vessels (Fig. 2 *e* and *f*).

Electron microscopic studies of the adrenal medulla and extra-adrenal ganglionic complex of NGF-injected fetuses in neonatal rats show that both consist of a mixed population exhibiting ultrastructural characteristics of immature and differentiated sympathetic cells. The coexistence in most of the cells of electron-dense vesicles indistinguishable from those of chromaffin cells demonstrates the origin and still incomplete transformation of these cells, which possess nerve and glandular features. A section of one of these cells, surrounded by axons and growth cones emerging from adjacent cells in the adrenal medulla of a 10-day-old rat, is reproduced in Fig. 3.

Discontinuation of the NGF treatment for a week results in the progressive degeneration of nerve cells in the medulla and in the extra-adrenal body adhering to the gland surface. The possible cause of death of the transformed nerve cells will be considered in the *Discussion*.

Light, fluorescence, and electron microscopic studies of AS-NGF-injected fetuses and neonatal rats

Fetuses injected on day 17 of gestation with the immunoglobulin fraction of a specific antiserum to NGF were killed on day 19 of pregnancy or were kept alive for 8 days after birth and injected daily with the same antiserum.

Histological studies performed on 19-day-old fetuses showed that differentiation lagged behind that of controls in the small cell aggregates interspersed between the epithelial cordons of the adrenal gland. In postnatal stages, pheochromoblasts and immature chromaffin cells showed signs of extensive degeneration in the cytoplasmic and nuclear compartments. They decreased progressively in number, leaving large empty spaces in the adrenal medulla which differed from that of controls also in the poorly developed vascular network. Fig. 4 shows a control and an experimental adrenal medulla in two 8-day-old rats. Most of the chromaffin cells have been wiped out. The disappearance of glandular cells that closely adhere to blood vessels has in turn produced the total disorganization and effacement of the medullary circulatory system (Fig. 4*b*).

Fluorescence microscopic studies in neonatal control and experimental rats showed an impressive difference in the spectral characteristics of adrenal medullary cells in normal and AS-NGF-injected rats. The former exhibit the typical intense yellow-green fluorescence, whereas the latter emit a pale orange-red color, indicating that synthesis and storage of catecholamines in chromaffin cells are severely altered.

Ultrastructural studies in 4-day-old control and experimental rats gave evidence of advanced signs of degeneration in the nuclear and cytoplasmic compartments of most adrenal medullary cells. Some cells, still fairly well preserved, showed accumulation of electron-dense vesicles in large aggregates, apparently resulting from coalescence of single vesicles. These findings give evidence for the degenerative effects produced by AS-NGF treatment initiated in fetal life not only on primi-

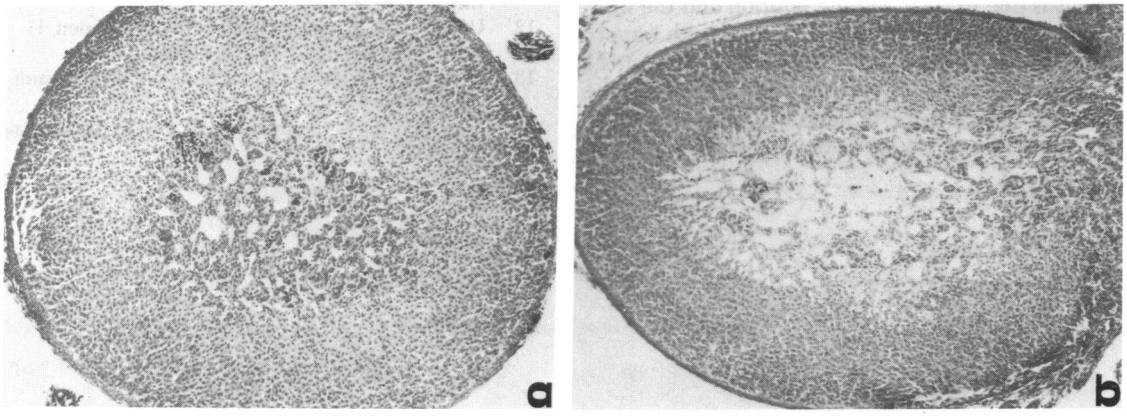

FIG. 4. Largest cross section of the adrenal gland of two 4-day-old rats injected pre- and postnatally with vehicle solution (*a*) or with a specific antiserum to NGF (*b*). Note in *b* almost complete disappearance of medullary cells and vascular network. (×60.)

tive sympathetic cells and pheochromoblasts, but also on cells that have already attained signs of differentiating into immature chromaffin cells.

DISCUSSION

The massive transformation of chromaffin cell precursors into sympathetic cells in the adrenal medulla of rats injected during fetal and post-natal life with NGF provides evidence that this molecule can divert glandular cells into nerve cells in the developing organism. Although both cell types originate from the same stem cells, they proceed at an early stage in their differentiation along two different lines. The end products—a glandular and a nerve cell—exhibit vast differences in their structural, biochemical, and functional properties. The NGF-induced transformation of chromaffin cells into sympathetic cells in the adrenal medulla has profound morphologic effects on the whole gland (see Fig. 2 *a* and *b*). Another effect of the fetal and postnatal treatment with NGF that calls for some comments is the formation of voluminous nerve-cell aggregates closely adhering to the medial surface of the adrenal gland. In control fetuses, small clusters of primitive sympathetic cells are seen in this same area, but they decrease in size toward the end of the fetal period and are no longer detectable in the first post-natal week. Their enormous increase in volume in experimemental rats could result from the NGF transformation of chromaffin cell precursors into sympathetic cells. Since differentiated cells are no longer endowed with migratory activity, they would permanently settle outside rather than inside the gland. At time of the first injection, a large number of migrating cells have already attained their destination and are lodged in the adrenal medulla, where they undergo proliferative activity. A NGF block of a late migrating wave would not result in any appreciable depletion of chromaffin cells in the fully differentiated organ.

Time-sequence studies from fetal to postnatal life showed a progressive decline of the NGF property to induce differentiation of presumptive chromaffin cells into sympathetic nerve cells. Fully differentiated glandular cells are no longer receptive to its action, whereas immature chromaffin cells undergo transformation into nerve cells but retain at the same time unequivocal signs of their original nature in the electron-dense vesicles seen in large number in the cell perikaryon. Discontinuation of the NGF treatment in the second or third postnatal weeks results in the massive degeneration of the transformed sympathetic cells. These findings raised the question of whether the *de novo* differentiated nerve cells do not substantially differ from sympathetic neurons, which obviously do not depend on exogenous NGF for their survival and further growth. An alternative, more plausible, explanation is that death of the transformed cells is due to their ectopic position and the impossibility of their axons establishing connections with target cells that would provide endogenous NGF sources. In favor of this hypothesis are the results of previous studies (8, 9) which proved that immature sympathetic nerve cells disconnected by chemical or surgical procedures from their end organs are doomed to death but survive and even undergo excessive growth when supplied with exogenous NGF.

The present *in vivo* experiments show that the range of action of NGF is not restricted to sympathetic nerve cells and embryonic sensory nerve cells (10), but extends also to chromaffin cell precursors and to immature chromaffin cells. Evidence for NGF transformation of a neoplastic cell line known as pheochromocytoma PC12 into sympathetic nerve cells *in vitro* was first presented by Greene and Tischler (11). More recently, Unsicker *et al.* (12) showed that chromaffin cells dissociated from the adrenal medulla of infant rats produce fiber outgrowth and exhibit structural, ultrastructural, and biochemical features of sympathetic cells when cultured in a NGF-enriched medium. The property of isolated adrenal medullary cells to occasionally produce neurites even in the absence of NGF had been shown in previous experiments (13, 14). The authors attribute the NGF effect to the fact that the dissociated chromaffin cells are not under the influence of glucocorticoids released in the intact gland from adjacent cortical cells. Addition to the culture medium of dexamethasone markedly reduced neuronal fiber outgrowth. In our studies *in vivo*, NGF injections channel chromaffin cell precursors toward the nerve cell-differentiating line and transform immature chromaffin cells into sympathetic cells even in the intact gland, thus overcoming the effects of glucocorticoid hormones. The devastating effects produced by injections of a specific NGF antiserum in the adrenal medulla of rats injected pre- and postnatally with this antiserum further add to the notion of a much broader role of NGF in the developing organism than was previously conceived.

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1. Levi-Montalcini, R. (1966) *Harvey Lect.* **60**, 217–259.
2. Angeletti, P. U., Levi-Montalcini, R., Kettler, R. & Thoenen, H. (1972) *Brain Res.* **44**, 197–206.
3. Bocchini, V. & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. USA* **64**, 787–794.
4. Levi-Montalcini, R. & Angeletti, P. U. (1961) *Q. Rev. Biol.* **36**, 99–108.
5. Levi-Montalcini, R., Aloe, L., Mugnaini, E., Oesch, E. & Thoenen, H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 595–599.
6. Coupland, B. (1965) *The Natural History of the Chromaffin Cells* (Longmans, Green, London).
7. Hervonen, A. (1971) *Acta Physiol. Scand. Suppl.* **368**, 1–94.
8. Aloe, L. & Levi-Montalcini, R. (1975) *Arch. Ital. Biol.* **113**, 326–353.
9. Hendry, I. A. (1975) *Brain Res.* **94**, 87–97.
10. Levi-Montalcini, R. & Booker, B. (1960) *Proc. Natl. Acad. Sci. USA* **46**, 373–384.
11. Greene, L. A. & Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2424–2428.
12. Unsicker, K., Krisch, B., Otten, U. & Thoenen, H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3498–3502.
13. Olson, L. & Malmfors, T. (1970) *Acta Physiol. Scand. Suppl.* **348**, 1–112.
14. Unsicker, K. & Chamley, J. H. (1977) *Cell Tissue Res.* **177**, 247–268.