Proc. Natl. Acad. Sci. USA
Vol. 77, No. 3, pp. 1656–1660, March 1980
Neurobiology

Intracellular distribution of nerve growth factor in rat pheochromocytoma PC12 cells: Evidence for a perinuclear and intranuclear location

(immunofluorescence/nerve growth factor antibodies/autoradiography)

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Communicated by Rita Levi-Montalcini, October 23, 1979

ABSTRACT **The distribution of nerve growth factor (NGF) in rat pheochromocytoma cells (clone PC12) has been studied with two different techniques: immunofluorescence and autoradiography. It was found that NGF is progressively internalized in the cytoplasmic compartment and eventually accumulates in the form of discrete dots around the nucleus. A fraction of the internalized NGF appears within the nucleoplasm, often contiguous with the nucleolus. It is suggested that cytoplasmic and perinuclear NGF may be in contact with a pool of tubulin or actin-like proteins in their soluble or organized form and play a key role in the process of arrest of division and neurite growth.**

Nerve growth factor (NGF) exerts an essential trophic and differentiating action on sympathetic and sensory neurons (1, 2). Recently a cell line, derived from a rat pheochromocytoma tumor and referred to as PC12 has been shown to respond to NGF by arrest of cell division, sprouting of electrically excitable processes, and transformation into a neuronal population having some biochemical and immunological properties similar to those of sympathetic nerve cells (3–5).

The multiple effects exerted by NGF in target cells—effects that in PC12 cells include some crucial steps of neuronal differentiation—raise the question of whether they are achieved through a unique mechanism or whether some (e.g., arrest of cell division and neurite growth) are mediated via distinct and independent actions of the ligand. An approach to this problem is to follow the fate of the effector molecules after interaction with their target cells and to localize their possible distribution within specific cell compartments.

In a previous series of studies it was found that a large portion of ^{125}I-labeled NGF (^{125}I-NGF) became tightly bound and resistant to trypsin digestion after binding to specific receptors in PC12 cells (6). Subsequent studies (unpublished data) indicated that tight binding of NGF is followed by its internalization into some cellular compartments.

In this paper we report the intracellular localization of this fraction of NGF, measured with two different techniques: immunofluorescence microscopy with monospecific antibodies and autoradiography after incubation with ^{125}I-NGF. These studies give evidence of an NGF localization not confined to the cytoplasm but also present in the nuclear compartment.

MATERIALS AND METHODS

Cells. Rat pheochromocytoma cells (clone PC12) were cultured at 37°C with RPMI-1640 (GIBCO) containing 10% heat-inactivated horse serum, 5% fetal calf serum, and 100 units of penicillin and 100 μg of streptomycin per ml; the atmosphere was 5.0% CO_2 in air. The cells used for immunofluorescence and autoradiography experiments were plated in 35-mm plastic tissue culture dishes containing round glass coverslips coated with polylysine. NGF was added to the medium at a concentration of 50 ng/ml.

Indirect Immunofluorescence Microscopy. Coverslips were removed and washed three times in serum containing medium and once in phosphate-buffered saline (P_i/NaCl). Coverslips were then fixed for 10 min in 3.7% formaldehyde in P_i/NaCl at room temperature, rinsed in P_i/NaCl, treated sequentially in prechilled methanol (−10°C) and acetone for 5 min and 5 sec, respectively, and finally dried in a gentle air stream. This treatment has been shown to make the plasma membrane permeable to antibodies and is widely used for immunofluorescence studies of intracytoplasmic proteins (7, 8). Monospecific NGF antibodies (30 μg/ml) were layered on coverslips and incubated for 45 min at 37°C. After rinsing, the coverslips were further incubated in fluorescein-labeled goat antibodies against rabbit IgG (1:50 dilution) for 30 min at room temperature. After several careful washings in P_i/NaCl the coverslips were mounted in Mowiol 4-80 (Hoechst, Frankfurt, West Germany) and viewed in the microscope. For each experiment, two sets of controls were run: one with cells not exposed to NGF and one in which the NGF antibodies were replaced with the same concentration of rabbit purified IgG.

All coverslips were observed in a Leitz Ortholux fluorescence microscope equipped with epi-illumination and an Orthomat automatic camera. Usually a Zeiss ×63 power planapochromatic oil-immersion objective was used.

Anti-NGF Antibodies. Antibodies against 2.5S NGF were raised in rabbits by using NGF purified from mouse submaxillary glands (9). Purified antibodies were prepared essentially by the procedure of Stoeckel *et al.* (10) based on affinity chromatography of the IgG fraction on Sepharose 4B with covalently linked 2.5S NGF. After absorption and washes as described, NGF antibodies were eluted with 4.5 M $MgCl_2$ and their immunological specificity was checked by immunodiffusion. Fluorescein-conjugated goat antibodies against rabbit IgG were obtained from Miles-Yeda, Rehovot, Israel.

Autoradiography. Cells plated on polylysine-coated coverslips were incubated in complete medium containing ^{125}I-NGF labeled according to Young *et al.* (11). ^{125}I-NGF (specific activity, 8–12 × 10^4 cpm/ng) was added to the culture medium at 1–2 × 10^5 cpm/ml with unlabeled NGF at 50 ng/ml. At different times of incubation the medium containing labeled NGF was removed and, after several rinses in prewarmed medium, the cells were further incubated for 2 hr in medium containing the same concentration of unlabeled NGF. With this

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Abbreviations: NGF, nerve growth factor; P_i/NaCl, phosphate-buffered saline.

procedure, all NGF specifically bound in a reversible fashion to the cell is released into the medium, leaving the portion tightly bound or internalized (6). Nonspecific binding of labeled NGF was measured by preincubating cells in a medium containing a large excess (10 μg/ml) of unlabeled NGF prior to the addition of the tracer.

Coverslips were rinsed in medium and P_i/NaCl and fixed in 3.7% (wt/vol) formaldehyde; after further rinsing and drying, the coverslips were dipped in Ilford K5 autoradiographic emulsion and exposed in the presence of dessicant for 3–8 days. After development and fixing, the coverslips were stained with buffered 0.5% toluidine blue, mounted, and photographed with a Leitz planachromatic ×100 oil-immersion objective.

RESULTS

Localization of NGF by Immunofluorescence Microscopy. Fig. 1 shows the staining of PC12 cells incubated with NGF for different times. These cells have specific binding sites for this growth factor which account for 88–92% of the total NGF binding; the remainder results from nonspecific binding or adsorption of the protein to the surface of the cells (6). Thus, we may infer that most of the monospecific antibody cross-reacting material shown in Fig. 1 represents NGF specifically bound to, embedded in, or internalized across the cell membrane. Time-course studies of NGF antibody localization show the presence of a diffuse intracellular fluorescence intercalated with fluorescent granules. Such fluorescence is only slightly detectable when cells are stained with preimmune serum (Fig. 1*f*) or with NGF antibodies but in the absence of NGF (see Fig. 2*f*). This faint, diffuse fluorescence is most probably due to the portion of antibodies nonspecifically adsorbed to the cytoplasmic membrane. After 1 hr (Fig. 1*a*) the fluorescent material is present in moderate amount and is mostly located in the flattened outer rim of the cells. Later, the diffuse and granular fluorescence increases considerably; between 6 and 24 hr (Fig. 1 *b–d*) the fluorescence concentrates in the area immediately surrounding the nuclear contour.

After 24–48 hr exposure to NGF an increasing high number of cells exhibit one or more brightly fluorescent dots apparently located within the nuclear area (Figs. 1*e* and 2). Accurate focusing and simultaneous observation in phase-contrast microscopy suggest that the fluorescent dots are perinuclear and intranuclear and very often are located contiguous to a large nucleolus. Fig. 2*a–e* shows some details of the intranuclear location of the NGF antibodies crossreacting material. After 1-day exposure to NGF, about 50% of the cells showed a bright nuclear dot and often two or three (Fig. 2*c*). The dots were smaller than the nucleolus but sometimes they were of the same size.

In order to assess whether the brightly fluorescent dots were indeed perinuclear and intranuclear and not merely adjacent to the nuclear outer surface, after incubation with NGF but before fixation, PC12 cells were incubated for 5 min in the presence of P_i/NaCl containing 0.5% of Nonidet P-40. Previous studies (unpublished observations) had shown that this treatment, followed by removal of medium and one gentle washing under the same conditions, removes 93–95% of total acetylcholinesterase activity, taken as a marker of cytoplasmic membrane proteins, but leaves most of the nuclei attached to the substratum. Staining of these nuclear preparations still

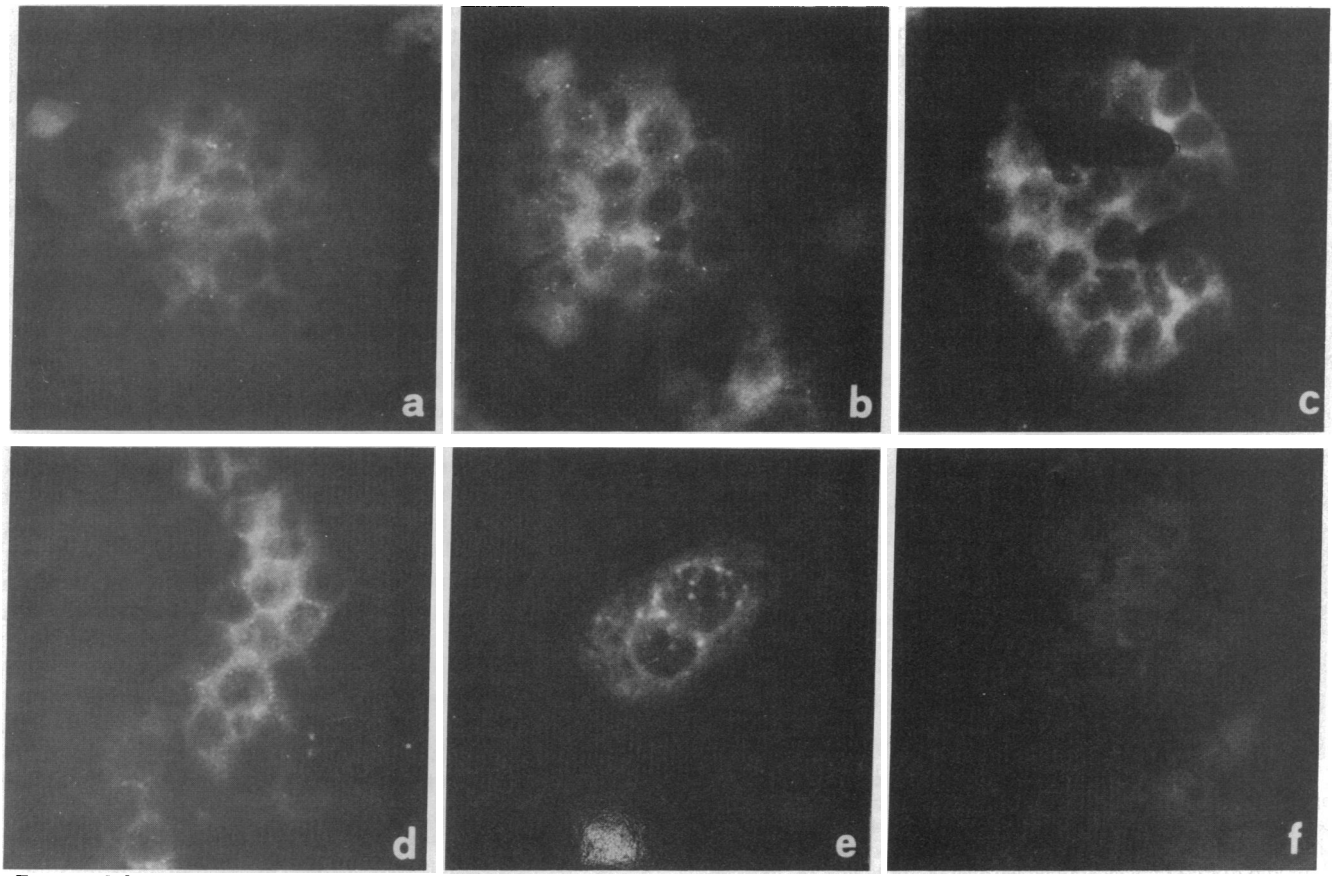

FIG. 1. PC12 cells were exposed to NGF for progressively longer times and processed for immunofluorescence microscopy with NGF specific antibodies. NGF exposure times were 1 hr (*a*), 6 hr (*b*), 12 hr (*c*), 24 hr (*d* and *f*), and 48 hr (*e*). In *f* the NGF antibody was replaced by the same amount of preimmune rabbit IgG. (*a–d* and *f*, ×550; *e*, ×630.)

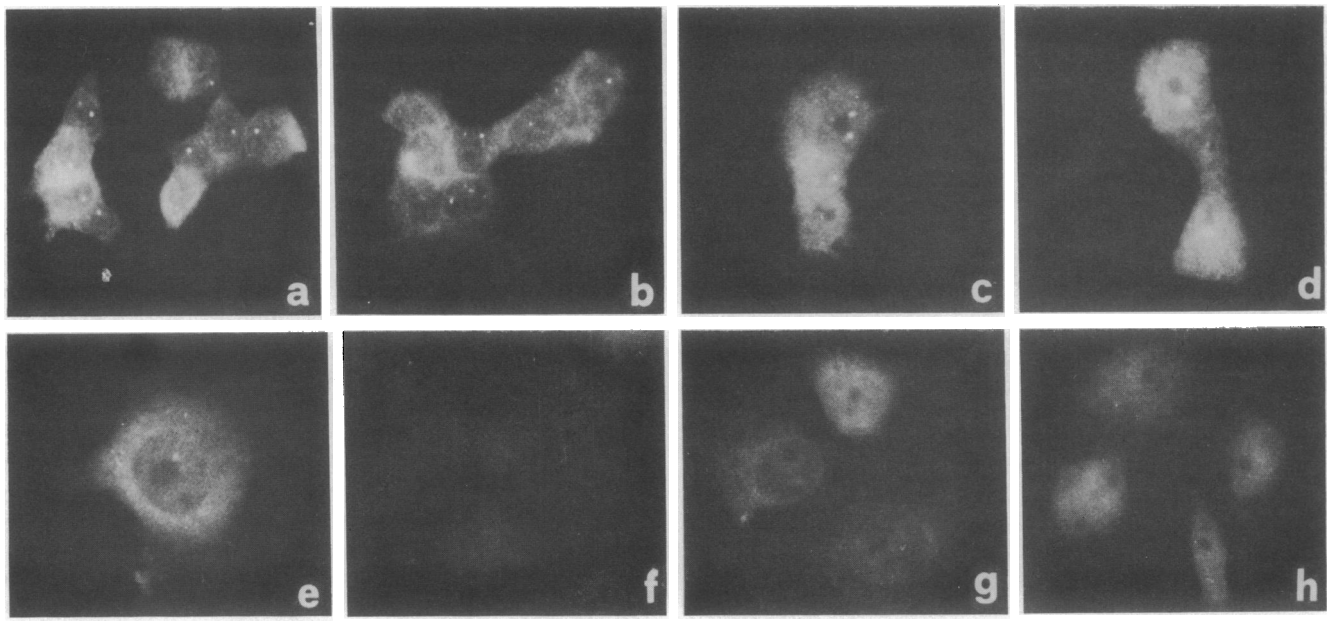

FIG. 2. PC12 cells were incubated with NGF for 24 hr (*a–d* and *g*) or 48 hr (*e*). Some cells (*f*) were grown in NGF-free medium. Others (*h*) were cultured for 48 hr in the presence of NGF, washed, and incubated in NGF-free medium for a further 48 hr. All the cells were processed for immunofluorescence microscopy by using NGF specific antibody except in *g* the NGF antibody was replaced by preimmune rabbit IgG. Controls of different types show a negative reaction (*f–h*); the details of the intranuclear localization of NGF immunoreactive material is shown in the experimental pictures (*a–e*). (*a* and *b*, ×550; *c* and *d*, ×660; *e*, ×630; *f–h*, ×600.)

showed the presence of fluorescent dots around the nuclear membrane and often in the nucleolar region (Fig. 3). Treatment of the cells for 2 hr with 1 μM colchicine did not alter the perinuclear distribution of fluorescent dots reported in Figs. 1 and 2. This finding indicates that if, as postulated in the *Discussion*, NGF is in contact with a pool of tubulin molecules and forms microtubule organizing centers, these complexes must be resistant to the action of microtubule poisoning drugs like colchicine. It is worth mentioning in this connection that, both *in vitro* and *in vivo*, NGF counteracts the destructive action of another antimicrotubular agent, vinblastine (12).

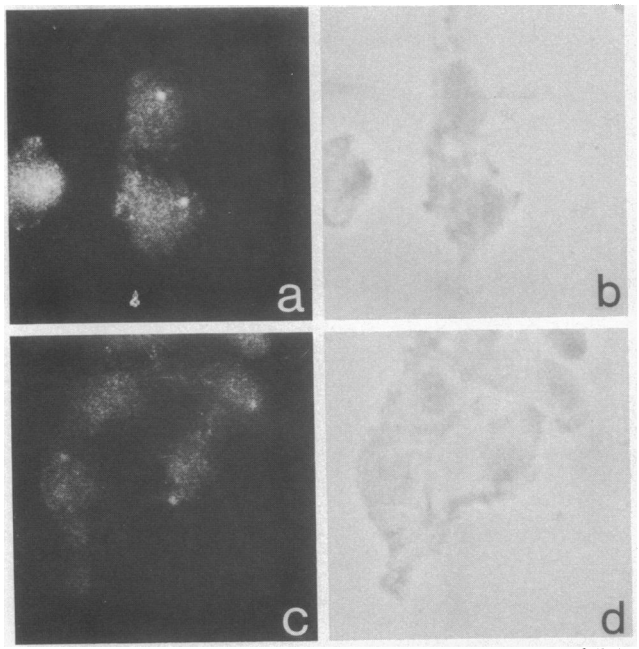

FIG. 3. PC12 cells were incubated in the presence of NGF (50 ng/ml) for 24 hr, washed, and further incubated for 5 min at room temperature in PBS containing 0.1% Nonidet P-40. After removal of this solution, nuclei were fixed and treated as described in Figs. 1 and 2. (*a* and *c*) PC12 nuclei stained with NGF antibodies (*a*) or preimmune serum (*c*). (*b* and *d*) Same nuclei visualized by phase-contrast microscopy. (×950.)

Intracytoplasmic and intranuclear fluorescence was not detectable under the following conditions: when cells had not been exposed to NGF (Fig. 2*f*); when they were incubated with NGF but stained with preimmune serum (Fig. 2*g*), and when, after exposure to NGF for 2 days, they were further incubated in its absence for 2 more days (Fig. 2*h*).

The distribution of ^{125}I-NGF assessed by autoradiography is shown in Fig. 4. Previous studies with ^{125}I-NGF had shown that approximately 50% of the total NGF bound to PC12 is dissociable and is released in the medium whereas the remaining portion is tightly bound to the membrane or internalized (6). In order to minimize the contribution of the portion of ^{125}I-NGF still bound in a reversible fashion to the receptors, after incubation with the labeled ligand but before fixation for autoradiography, cells were further incubated in NGF-free medium for 2 hr, a period sufficient to remove all reversibly bound NGF. Silver grains were absent from cells exposed to unlabeled NGF (Fig. 4*a*) or when the cells were incubated with ^{125}I-NGF in the presence of an excess of unlabeled NGF (Fig. 4*b*). Conversely, silver grains were found in the cytoplasmic and perinuclear areas after a 2-hr (Fig. 4*c*) or 6-hr (Fig. 4*d*) pulse followed by a 2-hr chase. Moreover, after 1–2 days of exposure to labeled NGF, a considerable number of cells showed silver grains over the nucleus, often in correspondence to the nucleolar outline (Fig. 4 *e* and *f*). The distribution of silver grains indicates a cytoplasmic location of the radioactive tracer during the first hours of incubation (Fig. 4*d*) followed by their spotty appearance also over the nuclear contour and in contiguity with the nucleolus (Fig. 4*f*). The nuclear location of grains was preserved even when, prior to fixation for staining, the cells were treated with trypsin (0.1 mg/ml, 10 min) or with Nonidet P-40, as described above, to remove the cytoplasmic membrane (not shown).

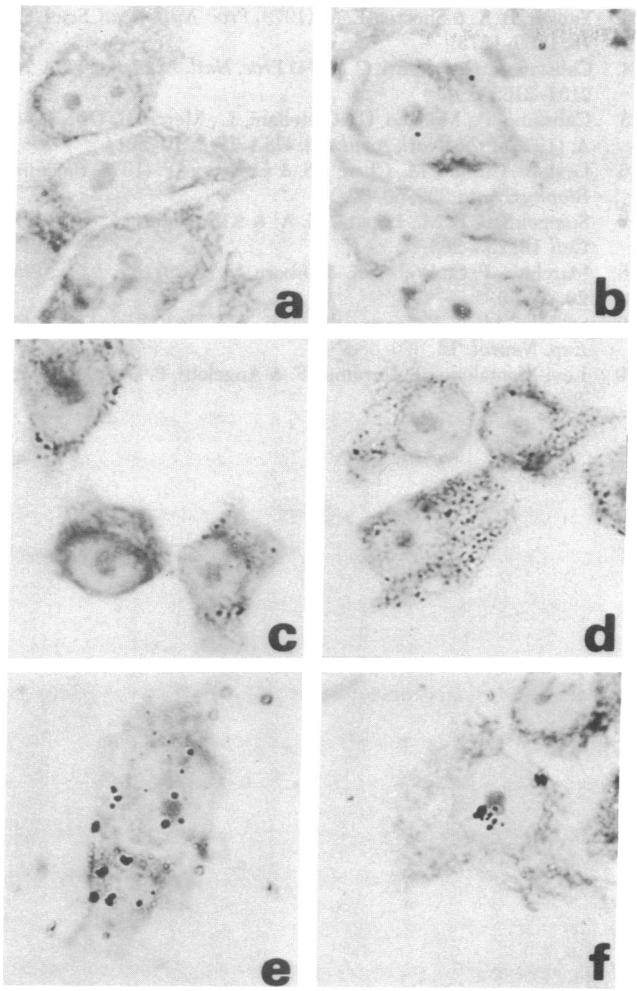

FIG. 4. PC12 cells were incubated with ^{125}I-NGF for 2 hr (*c*), 6 hr (*b–d*), and 24 hr (*e* and *f*) followed by 2 hr chase with unlabeled NGF. Some cells (*a*) were not exposed to labeled NGF; with others (*b*), the incubation was carried out in the presence of a large excess (10 μg/ml) of unlabeled NGF. Whereas controls (*a* and *b*) are almost devoid of silver grains, in the other cells grains are visible in the cytoplasm (*c* and *d*) and also in the nuclear area (*e* and *f*). (×1000.)

DISCUSSION

The distribution of NGF in pheochromocytoma cells (clone PC12) has been examined with two methods: immunofluorescence and autoradiography. The results strongly indicate that a portion of this protein is internalized into the cell cytoplasm and, by a few hours after its addition to the cells, becomes apparent in the perinuclear area and also within the nucleoplasm. A definite conclusion that autoradiographic silver grains and fluorescent spots are indeed in contiguity with the nucleolar outline must await further studies with improved resolution techniques. We consider however as strongly suggestive the present NGF localization because it has been obtained with two independent experimental approaches.

That the material responsible for immunofluorescence staining is indeed NGF, either native or a partially degraded but still immunoreactive fragment, is indicated by the following findings: (*i*) preimmune sera do not produce any detectable staining, (*ii*) the NGF antibodies do not stain cells not previously exposed to externally added NGF, and (*iii*) the fluorescence is not detectable when, after incubation of the cells with NGF, the cells are further incubated in NGF-free medium. Although the precise molecular mechanism responsible for NGF internalization is still obscure, we may infer that it is an active, energy-dependent event because it is completely abolished when cells are incubated with NGF at 4°C or when cells are exposed to various metabolic inhibitors (unpublished data).

The cytoplasmic and nuclear localizations of NGF are also demonstrated by autoradiography after incubation with ^{125}I-NGF. That the silver grains correspond to a pool of specifically internalized radioactivity is indicated by the lack of such grains when cells are incubated in the presence of an excess of unlabeled NGF. Preliminary experiments with electron microscope autoradiography under similar conditions confirm the distribution pattern described above (unpublished data).

While these experiments were in progress it was reported (13) that an increasing portion of ^{125}I-NGF bound to PC12 cells appears in a nuclear pellet isolated after detergent treatment of PC12 cells.

Although our experimental conditions differ from those of previous authors, our findings agree with their conclusions and, in addition, provide evidence that NGF gains access to the inside of the nuclear compartment and in close proximity of the nucleoli. Although the methods used in our studies are not suitable to quantitate the actual amount of NGF present in the perinuclear area and in the nuclear compartment, the intensity of the signals obtained in these experiments would indicate that the actual portion of NGF reaching such areas of the cell constitutes only a small fraction of the total intracellular or tightly bound NGF.

The experiments reported call for some comments on the biological significance of the intracellular distribution of NGF. The finding that the nuclear and perinuclear location of NGF is discrete, as revealed by the presence of some highly fluorescent spots, suggests that NGF molecules in these areas of the cells are not randomly scattered but rather are concentrated in clusters. It remains to be established whether this pool of NGF is still biologically active and what is the nature of the nuclear and perinuclear components to which it is bound. Our working hypothesis is that the NGF is in contact with a pool of tubulin or of actin molecules which have been shown in previous *in vitro* studies (14–16) to interact specifically with NGF and undergo some major physicochemical changes such as increased rate of assembly and organization into large bundles. The recent demonstration that the early molecular events that precede neurite growth in neuroblastoma cells are characterized by the appearance of several perinuclear organization centers for microtubule assembly (17, 18) lends support to the hypothesis that NGF localization described in this paper reflects its participation to form or modulate analogous nucleation sites in target cells. Alternatively or concomitantly, such pools of NGF molecules may play an as yet unknown role in modulating some nuclear function.

The finding that the earliest ultrastructural damages induced by NGF antibodies are located in the nuclear compartment (19, 20) particularly at the nucleolar level, suggests that whatever the role played by NGF in this area of the cell may be, it must be highly pertinent to its biological action.

We thank Prof. Rita Levi-Montalcini for stimulating discussion during the preparation of the manuscript. This investigation was partially supported by a Consiglio Nazionale delle Ricerche grant to P.C.M.

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