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Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Correlation with density of sympathetic innervation

(enzyme immunoassay/iris/atrium/submandibular gland)

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ABSTRACT **A two-site enzyme immunoassay is described which does not suffer from artifacts inherent in previous assays and has the necessary high sensitivity to determine the endogenous levels of nerve growth factor (NGF) in the sympathetic nervous system and its target organs. Monoclonal and affinity-purified polyclonal antibodies against mouse NGF (mNGF) were covalently linked to glass beads as the first site and coupled to the enzyme β-galactosidase as the second site. Detection of the fluorescent β-galactosidase reaction product permitted the determination of 0.01–0.02 fmol of mNGF per assay. The recovery of mNGF added to homogenates varied between 50% and 100%, depending on the tissue. Rat superior cervical and stellate ganglia were found to contain (mean ± SEM) 25 ± 4 and 19 ± 3 ng of NGF per g wet weight, respectively, and the densely innervated submandibular gland, heart atrium, and iris contained 0.5 ± 0.1, 1.0 ± 0.1, and 1.9 ± 0.3 ng of NGF per g wet weight, respectively. Heart ventricle and skeletal muscle, which are poorly innervated by the sympathetic nervous system, did not contain detectable levels of NGF (<0.3 ng/g wet weight). Serum contained <0.05 ng of NGF per ml. The correlation between NGF levels and density of innervation is consistent with the concept that the production of NGF in target organs determines their density of innervation by the sympathetic nervous system.**

Nerve growth factor (NGF) is a protein essential for the development and maintenance of function of the peripheral sympathetic nervous system (for a review, see ref. 1). However, evidence of the local synthesis of NGF in sympathetically innervated effector organs and of its function as a retrograde trophic messenger between effector organs and innervating neurons has only been indirect. (*a*) Exogenous NGF is taken up by a selective and saturable mechanism by sympathetic nerve terminals and is then transported retrogradely to the corresponding perikarya, where it evokes its characteristic biochemical effects (1, 2). (*b*) Interruption of the retrograde axonal transport by surgical or chemical procedures has the same effect as the neutralization of endogenous NGF by specific antibodies (1–3). Thus, during early stages of development, both interruption of retrograde axonal transport and neutralization of endogenous NGF by antibodies lead to a degeneration of the corresponding neurons. In differentiated neurons, the two procedures lead only to an impairment of function reflected by a decrease in levels of enzymes involved in the synthesis of the adrenergic transmitter norepinephrine.

Despite many attempts, it has not been possible to determine the levels of NGF in sympathetically innervated target tissues by reliable quantitative methods (4, 5). With bioassay techniques, NGF became detectable only if the tissues were denervated either *in situ* (6) or by being brought into culture (6, 7). This suggests that the levels of NGF in sympathetically innervated target tissues are extremely low because the locally synthesized NGF is rapidly removed by the sympathetic nerve terminals or because the terminals exert a negative regulatory influence on the synthesis of NGF. It therefore was essential to develop a reliable quantitative assay for determination of the extremely small quantities of NGF in the innervated target tissues.

We describe here the development of a sensitive two-site enzyme immunoassay (EIA) that can detect 0.2–0.5 pg of NGF per assay. Using this assay, we have determined the levels of NGF in rat sympathetic ganglia and in densely innervated target tissues such as the submandibular gland, the heart atrium, and the iris (8). In organs less densely innervated by the sympathetic nervous system, such as skeletal muscle and heart ventricle, the levels of endogenous NGF were below the detection limit of the assay.

MATERIALS AND METHODS

Principle of the Immunoassay. In order to avoid the pitfalls of competition assays (1, 4, 9), we used a two-site immunoassay. NGF is bound to a large excess of a first antibody covalently linked to a solid support and then is measured by the second β-galactosidase-labeled antibody.

Production of Antibodies. Affinity-purified polyclonal sheep antibodies against 2.5S mouse NGF (mNGF) were prepared according to the protocol of Stoeckel *et al.* (10). [We use the term "2.5S" to indicate the presence of a mixture of intact and terminal-octapeptide-cleaved βNGF (1).] Nonimmune sheep IgG (Nordic, Tilburg, Netherlands) was isolated by standard procedures (11). Monoclonal antibodies were prepared according to the procedure of Köhler and Milstein (12). BALB/c mice were immunized with 2.5S mNGF according to the procedure of Stähli (13). The mouse myeloma cell line Ag8.653 (14) was used for the fusion, and hybridoma clone 27/21 was used in all experiments. Antibodies were purified by adsorption to protein A-agarose. Nonimmune mouse IgG (serum obtained by cardiac puncture) and monoclonal antibodies against contaminants in standard preparations of bovine NGF were purified in the same manner.

Covalent Coupling of the Antibodies to the Solid Phase. Glass beads (1 mm in diameter, Sigma) were activated with 3-aminopropyltriethoxysilane according to the method of Weetall (15) and transferred to 1% glutaraldehyde/50 mM sodium phosphate, pH 7.3. After 3 hr at 4°C, the beads were washed six times with twice-distilled water. The antibodies were incu-

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Abbreviations: NGF, nerve growth factor; mNGF, mouse NGF; EIA, two-site enzyme immunoassay; SCG, superior cervical ganglion.

bated with the beads (400 per ml) overnight at a concentration of 100 μg/ml in 50 mM sodium phosphate buffer (pH 7.3) at 4°C. The remaining aldehyde groups were blocked by a 2-hr incubation at room temperature with the EIA buffer [50 mM Tris·HCl/0.2 M NaCl/10 mM $CaCl_2$/0.1% Triton X-100/1% gelatin (from calf skin, 60 bloom)/0.05% NaN_3, pH 7.0]. The beads were washed thoroughly with EIA buffer and stored in the same buffer at 4°C. The affinity of antibodies for mNGF was determined by equilibration of the solid-phase antibodies with 0.004–8 nM ^{125}I-labeled mNGF [iodinated according to the procedure described by Rohrer and Barde (16)]. Buffer, temperature, incubation time, and washing were identical to the first step of the EIA. Unspecific binding of ^{125}I-labeled mNGF did not exceed 5% of total binding. Scatchard plots of the specific binding were used for calculation of the affinity constants. The graphs were linear and the dissociation constants for the covalently bound antibodies were 0.2 nM for the monoclonal antibody and 0.3 nM for the polyclonal antibodies. Binding properties were not impaired during storage for up to 3 months in EIA buffer. The capacity of the covalently bound antibodies was 0.2 ng of mNGF per bead for monoclonal antibodies and 0.6 ng for sheep polyclonal antibodies.

Coupling of Antibodies to β-Galactosidase. The reaction was performed according to the method of O'Sullivan *et al.* (17), with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester used as crosslinker. Fifty percent of the antibody and 60% of the β-galactosidase (Boehringer Mannheim, 880 units/mg of protein) were crosslinked according to densitometric analysis of a Coomassie blue-stained native 1% agarose gel [pI values: β-galactosidase, 4.6; IgG, 5.8–7.3 (18, 19)]. Eighty percent of enzyme activity was retained after coupling. No loss of enzyme activity was observed in up to 1 year of storage at 4°C in 10 mM sodium phosphate/50 mM NaCl/1% bovine serum albumin/0.05% NaN_3, pH 7.0.

Preparation of Samples for EIA. Wistar rats (100–200 g, both sexes) were killed by cervical dislocation. Blood was collected and EDTA and aprotinin were added to final concentrations of 5 mM and 100 kallikrein units/ml, respectively. Organs were rapidly removed and frozen on dry ice until used. It proved to be essential, particularly for the iris, to avoid contact with saline during dissection because then NGF was lost to varying extents by diffusion. The tissues were homogenized with Kontes or Duall homogenizers (glass/glass, 10–20 strokes) at 0°C in 4–50 vol of 50 mM Tris·HCl/2% bovine serum albumin/2% gelatin/0.4 M NaCl/2 mM EDTA, pH 7.0, containing aprotinin (20 kallikrein units/ml), 0.1 mM phenylmethanesulfonyl fluoride (Merck), 0.1 mM benzethonium chloride (Serva, Heidelberg, Federal Republic of Germany), and 1 mM benzamidine. The homogenates were centrifuged for 3 min at $10^5 \times g$ at room temperature (Beckman Airfuge) and the supernatants were diluted 1:1 with 20 mM $CaCl_2$/0.2% Triton X-100.

The resulting samples were then tested in the EIA with both polyclonal sheep and monoclonal mouse antibodies to mNGF. Nonimmune sheep and mouse IgG fractions and two monoclonal antibodies against contaminants in standard preparations of bovine NGF (molecular structure unknown), covalently linked to glass beads in an identical manner as the NGF-specific antibodies, were used for evaluating the background signal of samples. NGF-specific beads and nonspecific beads had essentially the same background signal when tested in the buffer used for preparation of samples. However, the background signal of the nonspecific beads was 2- to 10-fold higher (depending on the tissue) when they were incubated with samples, which would correspond to the equivalent of up to 0.05 ng of NGF per ml. This false-positive signal therefore had to be subtracted from the total signal of samples tested on NGF-specific beads. Because, in the two-site immunoassay, antibodies are used in large excess over the antigen, an unspecific but kinetically stable adsorption of sample components to antibody molecules is most probably responsible for the positive signal that becomes apparent owing to the sensitivity of the assay.

Samples were considered to contain NGF only when the specific signal was at least 50% above the background and only when such a signal was obtained for both polyclonal and monoclonal NGF antibodies.

Performance of the EIA. Assays were carried out with either monoclonal or polyclonal antibodies; the same antibody was used for the first and second site. (This approach is possible because NGF is a homo-dimer.) One antibody-coated glass bead per well of a microtiter plate (Greiner, Nürtingen, Federal Republic of Germany) was incubated overnight at room temperature with 50 μl of sample or mNGF standard solution (buffer composition identical to that of the samples) in a water bath shaking at 20 rpm. The beads were washed twice with 200 μl of EIA buffer; 50 μl of antibody–β-galactosidase conjugate (diluted 1:300 in EIA buffer/1% bovine serum albumin, corresponding to 50 milliunits of β-galactosidase per bead) was added per well and incubated for 2 hr at 37°C and 20 rpm. The beads were washed twice with 200 μl of EIA buffer and once with 150 μl of 50 mM sodium phosphate/10 mM $MgCl_2$, pH 7.3, and then transferred to 3.5-ml polystyrene tubes (Greiner) each containing 50 μl of 100 μM 4-methylumbelliferyl-β-D-galactoside in 50 mM sodium phosphate/10 mM $MgCl_2$, pH 7.3. The enzyme reaction was linear up to 30 hr and was terminated after 15–25 hr at 37°C in the dark by addition of 650 μl of 0.15 M glycine·NaOH (pH 10.3). The fluorescent 4-methylumbelliferone formed was measured (364 nm excitation and 448 nm emission) in a Perkin–Elmer fluorometer (model 650-40). Detection limit was 0.1 ng of 4-methylumbelliferone per ml. Values were measured in quadruplicate. A standard curve in the range of 2.5 pg to 1.28 ng of mNGF per ml was determined for each assay.

Determination of Biological Activity of NGF. The biological activity was estimated by using the plasma clot bioassay of Fenton (20). Ten to 15 superior cervical ganglia (SCG) were homogenized in 150 μl of Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal calf serum, 2% bovine serum albumin, 2% gelatin, 0.1 mM leupeptin, aprotinin at 20 kallikrein units/ml, penicillin at 200 units/ml, and streptomycin at 200 units/ml. The homogenate was centrifuged for 3 min at $10^5 \times g$ (Beckman Airfuge), and the supernatant was used undiluted in the bioassay. Where appropriate, affinity-purified sheep antibodies (10 μg/ml) were added to the supernatant. The standard curve for mNGF was determined in the same medium.

Reagents. If not otherwise stated, all reagents were obtained from Sigma.

RESULTS

EIA Sensitivity and Specificity. The EIA could detect mNGF down to 5 pg/ml (0.2 pg per assay) reproducibly with the monoclonal antibody 27/21 and down to 10 pg/ml (0.5 pg per assay) with polyclonal antibodies, corresponding to 0.01 and 0.02 fmol of mNGF/assay (Fig. 1). Signal-to-blank ratio at the detection limit was 1 in the standard curve. When NGF was added to homogenates or blood at concentrations between 0.05 and 0.2 ng/ml, the recovery of NGF was 50% to 100%, depending on the tissue examined (Table 1). Several substances that resemble NGF in either isoelectric point or molecular weight (cytochrome *c*, aprotinin, β-bungarotoxin) or have partial sequence homologies (insulin) gave no signal when tested in the EIA at a concentration of 10 μg/ml.

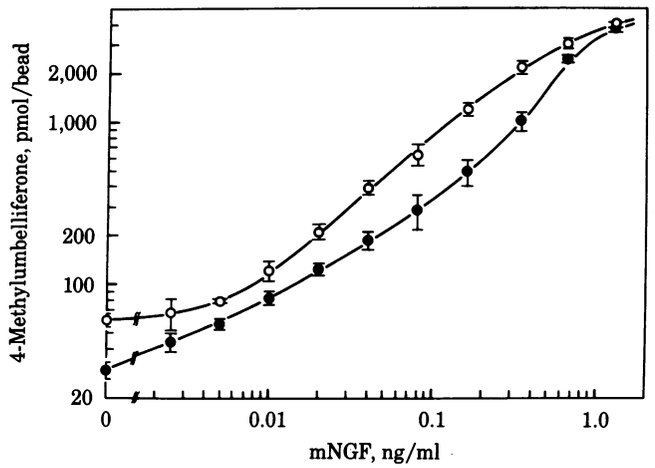

FIG. 1. Standard curves of the EIA for mNGF. Bound β-galactosidase activity, expressed as pmol of liberated 4-methylumbelliferone, as a function of mNGF concentration. ○, Polyclonal sheep antibodies, 24-hr incubation with 4-methylumbelliferyl-β-D-galactoside; ●, monoclonal antibody, 15-hr incubation with 4-methylumbelliferyl-β-D-galactoside. Values are means ± SEM.

NGF Content of Rat Tissues. NGF concentrations are expressed in mNGF equivalents. NGF levels were determined with monoclonal and polyclonal antibodies. Both methods rendered essentially the same results (no significant difference, $P < 0.9$). Therefore, the data for both kinds of antibody were combined for each tissue. No significant differences between NGF tissue contents in males and females were found ($P < 0.7$). NGF concentrations were highest in SCG and stellate ganglia, but NGF was also detectable in submandibular gland, atrium, and iris (Table 1). The NGF content of heart ventricle and skeletal muscle was too low to be measured (<0.3 ng/g wet weight), and no NGF was found in the serum (<0.05 ng/ml). For SCG, the tissue with the highest NGF concentration measured by EIA, estimation of NGF content was also possible by the chicken dorsal root ganglia bioassay (20). However, because concentrated homogenates had to be used, the evaluation was difficult (cloudiness of the plasma clot) and consequently the scattering of the biological response was high, ranging between 0.002 and 0.01 biological unit per SCG, corresponding to 10–70 pg of NGF per SCG. Neurite outgrowth was completely blocked by affinity-purified sheep polyclonal antibodies at 10 μg/ml.

Table 1. NGF content* in rat organs *in vivo*

Organ	NGF† ng/g wet weight	NGF† pg/organ	Recovery of added mNGF, %‡
SCG	25 ± 4	42 ± 7	86
Iris	1.9 ± 0.3	1.7 ± 0.3	84
Submandibular gland	0.5 ± 0.1	85 ± 11	110
Stellate ganglion	19 ± 3	31 ± 5	100
Heart atrium	1.0 ± 0.1	14 ± 2	50
Heart ventricle	<0.3	<150	46
Skeletal muscle	<0.2	—	47
Serum	<0.05 ng/ml	—	69

* Measured as mNGF equivalents. Because the NGF contents of male and female rats were not significantly different ($P < 0.7$), results from both sexes were pooled. The data are corrected for the corresponding recoveries.

† Values are means (± SEM) of at least four independent determinations.

‡ mNGF was added in the range of 0.05–0.2 ng/ml, so that the signal of the sample was roughly doubled.

DISCUSSION

The information on NGF levels in tissues and blood has been obscured by results of inappropriate assay methods. High levels of NGF have been determined by various competition assays (21–25). The results of these assays are erroneous, however, because NGF-binding macromolecules—of which one has been identified in serum (9)—compete with the binding sites of antibodies or receptor preparations for labeled NGF and therefore mimic the presence of high levels of endogenous NGF (for detailed discussion see refs. 1, 4 and 9). Two-site immunoassays avoid such pitfalls. There, the NGF to be determined is bound to a large excess of antibodies linked to a solid phase and is then quantified by the second antibody which is radio- or enzyme-labeled. The accuracy and reliability of the two-site assay depend essentially on the quality of the antibodies—i.e., the use of monoclonal or affinity-purified polyclonal antibodies against NGF is imperative (cf. 26). The previously available two-site assay that took these criteria into account was not sufficiently sensitive to detect endogenous NGF in sympathetic ganglia and their target organs (4).

We therefore developed a two-site immunoassay that is about 500-fold more sensitive than the previous one. This increase in sensitivity results mainly from (*a*) coupling of the second antibody to β-galactosidase, leading to a much higher signal compared to that from ^{125}I-labeled antibodies and (*b*) covalent coupling of the first antibody to glass beads to allow a more rigorous washing (including detergents) without loss of NGF–antibody complexes, resulting in an increase in the signal/background ratio. A limitation of the two-site immunoassay remains, however, owing to the restricted crossreactivity between antibodies against mNGF and NGF of other species. For example, the immunological crossreactivity between mouse and bovine NGFs in the two-site immunoassay is only about 10% (27).

In order to avoid a possible contamination of the tissue samples by the extraordinarily large quantities of NGF present in the mouse submandibular gland, we performed our experiments in rats. The efficient immunosympathectomy in rats induced by anti-mNGF antibodies indicates that the immunological crossreactivity with rat NGF is good (3). Additionally, to avoid false-negative results due to a partial lack of immunological crossreactivity, we performed parallel determinations with monoclonal and affinity-purified polyclonal antibodies. The monoclonal antibodies have the advantage of absolute specificity for a given epitope, but this single antigenic site might also be present on molecules unrelated to NGF or might be absent on NGF molecules of other species. The polyclonal antibodies lack this absolute specificity, recognizing instead an average of multiple antigenic sites. Because the values determined with the monoclonal and polyclonal antibodies did not differ significantly from each other, we conclude that the mNGF equivalents determined in rats represent a relatively accurate measure of the quantity of rat NGF. This is further supported by the observation that, in the SCG, which had the highest NGF content of all the rat tissues examined, the quantity measured by immunoassay was in agreement with the values found by the semiquantitative classical bioassay using chicken dorsal root ganglia.

In view of the limited immunological crossreactivity between the NGF molecules of different species, the biological assays in principle would have the advantage that the response depends on the active site of the NGF molecules, highly conserved during evolution (see refs. 1 and 27). However, in addition to the insufficient sensitivity (5) and the semiquantitative nature of the bioassays, limitations of specificity also have to be taken into account: macromolecules unrelated to NGF can

evoke similar responses. For example, a molecule recently purified from brain has been shown to support the survival of chicken sensory neurons and to promote neurite outgrowth like NGF (28). Other factors support the survival or promote neurite outgrowth from chicken sympathetic neurons and PC12 cells (29, 30). Therefore, as a criterion of specificity, it is necessary that the biological response to the putative NGF molecule be blocked by specific antibodies against NGF, and thus the bioassays are also restricted by the limited immunological cross-reactivity. Moreover, evaluation of a small contribution of NGF in a complex mixture of growth-promoting activities is extremely difficult. For example, in rat sciatic nerve only about 30% of the neurite growth-promoting activity is (perhaps) attributable to NGF, the rest being unaffected by NGF antibodies (31).

The increased sensitivity of the present two-site immunoassay allows quantitative determination of the NGF content not only in rat sympathetic ganglia but also in the corresponding target organs. These concentrations are one to three orders of magnitude lower than those previously reported for rat and mouse tissues on the basis of competition assays (21–24), two-site immunoassays with impure antibodies (32), or bioassays without adequate antibody controls (33). The NGF concentration in rat serum is still below the limit of our assay (<0.05 ng/ml). Because the concentrations of NGF necessary for survival and selective enzyme induction in rat sympathetic neurons *in vitro* are orders of magnitude higher (34, 35) than the level found in serum, the extremely low concentrations of circulating NGF are unlikely to play any physiological role and therefore the use of the term "hormone" for NGF is inappropriate.

The quantities of NGF found in sympathetic target tissues cannot be used to determine the actual concentrations at the nerve terminals because NGF present in the target tissues would be expected to be released into the extracellular space, according to the results of tissue culture experiments (6). The actual volume available for the distribution of NGF is probably much smaller than the total volume of the tissue, so that the effective NGF concentration at sympathetic nerve terminals would be expected to be much higher than the tissue concentrations found. The levels also most probably represent the end result of production and removal of NGF via retrograde axonal transport. This interpretation is strongly supported by the observations that the NGF content of the rat iris increased about 30-fold when the tissue was in culture for 24 hr (unpublished data; cf. ref. 6) and that the highest levels of NGF are present in sympathetic ganglia which accumulate NGF by retrograde axonal transport (see ref. 1). The local production of NGF by non-neuronal cells in sympathetic ganglia does not seem to play a major role because the interruption of the retrograde axonal transport of NGF cannot be compensated for by local production in the ganglion (1, 2).

The NGF levels in various target organs are correlated with the density of innervation (8), suggesting that density of innervation may be controlled by differences in the synthesis of NGF. This is in agreement with the increase in sympathetic nerve fiber production *in vivo* after local NGF injection (36) and the recent observation by Campenot (37) that NGF concentrations above those necessary for the survival of sympathetic neurons enhance the density of fiber outgrowth *in vitro*. The mechanism(s) that regulate the extent of NGF synthesis remain to be established.

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