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Characterization of nerve growth factors (NGFs) from snake venoms by use of a novel, quantitative bioassay utilizing pheochromocytoma (PC12) cells overexpressing human trkA receptors

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This paper is dedicated to Prof. Anthony T. Tu, to honor his retirement and his contribution to snake venom research and continuous support and encouragement of colleagues from all over the world to study snake venoms.

Abstract

Snake venoms are a very abundant source of nerve growth factors (NGF). NGFs of *Elapidae* showing 65% sequence homology with mouse or human NGF, while the *Viperidae* NGF shows *N*-glycosylation (Asn-21) typical of these mammalian NGFs. Snake NGF-induced neurite outgrowth (neurotropic activity) was measured in the past by using PC12 cell or dorsal root ganglion bioassays.

The present study was aimed at comparing, by dose-response experiments, the neurotropic activity of cobra and vipera versus mammalian NGFs, by using a novel bioassay involving PC12 cells genetically engineered to overexpress NGF-trkA receptors of human origin.

These cells respond to NGF by differentiation (morphologically expressed as neurite outgrowth) by a process mediated by NGF-trkA receptors. This process was evaluated by two different criteria: (1) elongation of neurites (E), and (2) Percentage of responsive cells (PRC) determined by digital acquisition of data and computer analysis. We found that snake venom NGFs were

Abbreviations: NGF, nerve growth factor; m, mouse; rh, recombinant human; PC12, pheochromocytoma cells; trkA, NGF high-affinity receptor; nnk, *Naja naja kaouthia*; vrr, *Vipera russelli russelli*; NCBI, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov); MW, molecular weight; S, sedimentation velocity unit under centrifugation; PRC, percentage of responsive cells; E, elongation of neurites; PC12wt, PC12 wild-type clone, expressing low level of trkA receptor; PC12-6.24-I, PC12 clone overexpressing rhtrkA-receptor; EC_{50}, effective concentration 50%; SEM, standard error of the mean.

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[†] Shortly after submission of this manuscript, Prof. Jashovam Shani passed away. His many colleagues will long remember him not only for his scientific contribution but also for his gentle guidance, friendship and humble humanity.

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less potent than mouse NGF, and that cobra NGF was more potent than vipera NGF. These data indicate the following order of NGF activity towards recombinant human trkA receptors: recombinant human NGF > mouse NGF > cobra NGF > vipera NGF. The neurotropic efficacy of these NGFs was found to be similar, reaching 80–90% of maximal activity obtained with all NGF forms. Interestingly, cobra (but not vipera) NGF demonstrated prolonged neurotropic activity compared with mouse NGF.

The results of the present study indicate that cobra and vipera venom NGFs represent natural agonists of human trkA-receptor of a lower potency, but of similar efficacy, compared with mammalian NGFs. These compounds are important pharmacological tools to characterize the trkA receptor structure-function relationship, and to develop novel neurotropic drugs.

Keywords: Mouse; Human; Cobra; Vipera; Nerve growth factor; Bioassay; PC12; trkA-receptor; Overexpressor; Potency; Efficacy; Neurite outgrowth; Stability; Digital

1. Introduction

Since having first been discovered by Cohen and Levi-Montalcini (1956), snake venoms have been considered a rich source of nerve growth factor (NGF) (Kostiza and Meier, 1996). Snakes are divided into three families: *Elapidae*, *Viperidae*, and *Crotalidae*. The venoms of all snakes belonging to these families contain different isoforms of NGF (Hayashi et al., 1996; NCBI protein data bank). *Elapidae* NGF has been isolated from *Naja naja* from different subspecies such as *Naja naja siamensis* (Selby et al., 1987; Inoue et al., 1991) and *Naja naja atra* (Oda et al., 1989; Inoue et al., 1991). These NGFs of a known sequence differ in 40 out of 118 amino acids of the β-chain, the chain which is solely responsible for the NGF neurotropic activity (Ulrich et al., 1983). The majority of research on NGF has been performed using m-NGF isolated from the male mouse submaxillary gland (NCBI, NP38637), and more recently also on rhNGF (NCBI, AAA59931). NGF is produced by the murine submaxillary gland as a precursor complex of about 130,000 Mw complex (also named 7S). This complex is composed of 3 subunits: α, β and γ, which disassociate at a very acidic pH, enabling the isolation of the active β-subunit (Hayashi et al., 1996). The β-subunit, the so-called β-NGF (2.5S), is the active subunit of NGF and exhibits all of the biological activities classically described for NGF, e.g. neuronal survival, proliferation, and differentiation (morphologically expressed as neurite outgrowth; Moore et al., 1974). Although 7S-NGF, like 2.5S-NGF, induces neurite outgrowth from PC12wt cells, there are differences in biological activity between the two factors (Shao et al., 1993). The route for biosynthesis of snake venom β-NGFs is not known, but it is assumed that they are derived from a larger precursor that is processed by endopeptidases, which are highly abundant in snake venoms (Kostiza and Meier, 1996).

β-NGF belongs to a family of neurotrophic factors that regulate the survival and differentiation of neurons in the peripheral and central nervous systems (Barbacid, 1995). β-NGF plays a major role in the survival and differentiation of certain cholinergic neuronal pathways in the brain (Fujita et al., 1989). NGF is also responsible for the survival of the neurons of the sympathetic and sensory nervous systems (Levi-Montalcini, 1987). It acts, like other neurotrophins, by binding to and activating receptor tyrosine kinases that are encoded by the TRK gene family (Kaplan and Stephens, 1994; Jiang et al., 1997). Interest in NGF and in its receptors has increased in the last decade (Oshima et al. 1996), due to discovery of therapeutically potential human-NGF (Perez-Polo et al., 1983) in neurodegenerative disorders (Gao et al., 1997; Swanson et al., 1998; Brade, 1989). Furthermore, developments in biotechnology made possible the production of highly pure NGF, including rhNGF of high standard quality for industrial uses (Fujimori et al., 1992; Ernfors et al., 1989) and clinical purposes (Tuszynski and Gage, 1990; Fahnestock et al., 2001).

Pheochromocytoma is a rare tumor that rises from chromaffin cells of the adrenal medulla. As there is no available human cell line of pheochromocytoma growing in vitro, the most established, well-characterized, in vitro model to explore NGF activity is the rat pheochromocytoma PC12 cell line, originally developed over 25 years ago from the adrenal medulla of an NEDH rat (Greene and Tishler, 1976). NGF promotes the differentiation of these pheochromocytoma cells into cells that acquire characteristics of sympathetic neurons, thus making PC12 cells a good model for studying the activity of NGF, as well as that of other neurotrophic factors (Fujita et al., 1989). Following exposure of PC12 cells to it, NGF activates its specific cell-surface trkA receptors, and long-term transcriptionally mediated events, resulting in proliferation arrest and induction of differentiation, morphologically expressed as neurite formation (Vaudry et al., 2002). Wild-type PC12 cells have been used for several decades as a bioassay for NGF (Greene, 1977). These cells express a low level of trkA receptors per cell, resulting in a slow (several days) time-course of NGF differentiation, which is disadvantageous for practical bioassay purposes. Therefore, we improved that assay by using a stable transfectant of PC12 cells that overexpress trkA receptors (Katzir et al., 2002). The advantage of this novel bioassay is the fast extension of the neurites which can

be evaluated after two days of NGF treatment instead of after seven days as required with the wild-type PC12 cells (Katzir et al., 2002). This novel bioassay was employed in the present study to characterize snake NGF neurotropic activity in comparison with mammalian 2.5S-NGF (mouse and human) and of 7S-NGF (mouse) precursor isoforms. To speed up the bioassay measurements, we also introduced a novel computerized approach and analyzed it versus the manual method. The present study characterizes cobra and vipera venom NGF as novel rhtrkA agonists to facilitate neurotropic drug development and pharmaceutical research of the trkA receptor.

2. Materials and methods

2.1. Materials

Rat tail collagen type 1, media, and their supplements (antibiotics and serum) used for cell culture were purchased from Biological Industries, Beit-Haemek, Israel. Various tissue culture dishes were purchased from Nunc, Denmark or Falcon, USA.

2.2. Preparations of NGF

Mouse β-NGF used for this study was purified from murine submaxillary glands (2.5S-mNGF) according to the procedure described earlier (Bocchini and Angeletti, 1969), lyophilized, and supplied by Alomone Labs. Jerusalem, Israel. Mouse 7S-NGF was purified from the mouse submaxillary gland according to the procedure described by Varon et al. (1967) and supplied by Alomone, Jerusalem, Israel. *E. coli*-derived human recombinant NGF was kindly supplied by Alomone Labs, Jerusalem Israel.

Snake venom NGFs were purified from the lyophilized venoms (The Japan Snake Institute) of *Naja naja kaouthia* and *Vipera russelli russelli* by a method similar to those described previously (Oda et al., 1989; Inoue, 1991; Koyama et al., 1992). The venoms were dissolved in 1% acetic acid and fractionated on a Sephadex G-50 column (3.2 × 100 cm) that had been equilibrated with the same solvent. The fractions containing NGF were lyophilized, dissolved in 0.05 M acetate buffer (pH 5.0), and applied on an S-Sepharose Fast Flow column (1.5 × 16 cm) that had been equilibrated with the same buffer. The adsorbed NGFs were eluted with the buffer containing a linear increasing concentration gradient of NaCl from 0 to 2 M. The NGF fractions thus obtained were further purified by reversed-phase HPLC, on a Vydac protein C4 column (4 × 200 mm) with 0.1% trifluoroacetic acid containing a linear increasing gradient from 0 to 80% acetonitrile. The N-terminal 10 amino acid residues of the purified NGFs were determined by Procise model 491HT protein sequencer and confirmed to be identical with those reported previously (Hayashi et al., 1996). Finally, 1.1 mg of *N. naja kaouthia* NGF and 0.5 mg of *Vipera russelli russelli* NGF were obtained from 2 and 1 g of the lyophilized venoms, respectively.

2.3. PC12 cell clones

PC12 wild-type clone was originally supplied to us by Dr G. Guroff (NIH, MD, USA). The cells were grown in DMEM supplemented with 7% fetal bovine serum, 7% horse serum, 100 μg/ml streptomycin and 100 U/ml penicillin. The cells were maintained at 37 °C in a 6% CO_2 incubator, with medium being changed twice a week. Cell cultures were split at a ratio of 1:6 once a week (Katzir et al., 2002). PC12-6.24-I overexpressing trkA receptors, a subclone developed in our laboratory from PC12-6.24 cells (Hampstead et al., 1992) were maintained under similar conditions in the presence of G-418 (200 μg/ml) to ensure stable expression of the plasmid encoding the trkA receptor gene (Katzir et al., 2002)

2.4. Bioassay setup

PC12 cells, at a density of 10,000/ml, were plated in 24-well culture dishes, pre-coated with 200 μg/ml collagen, and grown for 24 h in the respective medium before the actual experiment. To start the bioassay, we replaced the medium in each well with fresh medium containing the test NGF at an appropriate concentration, in a constant volume of 1 ml, and then incubated the plates for a period of 2 or 7 days (Katzir et al., 2002). The optimal density of the cell suspensions was adjusted to 5000–10,000 cells per ml. At this density the neurite outgrowth of the PC12 cell clones is not disturbed by lack of space between cells. In the cultures exposed for 7 days, the medium was changed twice. Every concentration of NGF was tested in duplicate, and each experiment was repeated three times. Two pictures were taken of each well at random spots. In each picture at least 25 cells were evaluated. Altogether, at least 100 cells were measured for each NGF concentration. The diameters of the PC12 cells ranged from 9 to 17 μm. The length of the neurite outgrowth progressively increased from less than 1 cell-diameter, and reached up to 10-fold the cell diameter. We defined an outgrowth length of 2-cell diameters as the minimal biological response to be considered in the calculation of our morphological differentiation parameters. This value was determined upon observation that even in the absence of NGF treatment, the different PC12 clones, on collagen-coated substrate initiated spontaneous neurite outgrowth. Two parameters were calculated: (1) Percentage of responsive cells (PRC), defined as the percentage of cells with neurites, out of all cells in the frame, and (2) Elongation of neurites (E), defined as the ratio between the neurite length and the cell diameter. A ratio of less than 2.0 indicated a lack of NGF-induced differentiation, and was not considered in the results.

2.5. Light microscopy and photography

All pictures for the quantitative measurements were taken at × 250 magnification, and the measurements of neurite length were made using a micrometer scale of an Olympus MI2 camera attached to an Olympus inverted light microscope (Olympus, Hamburg, Germany). Pictures were printed from 35-mm Ilford black-and-white negatives of a 125 ASA film. Illumination conditions were kept constant. Selected photographs at high resolution were also taken at × 100 magnification.

2.6. Evaluation methods

Three evaluation methods were compared in this study: (1) Manual performance of measurements, using directly printed photographs; (2) Computerized measurements by a morphometric system manufactured by Galai Electro-optic Laboratories for Research and Diagnosis Ltd (Migdal-Haemek, Israel), model C-3; and (3) Computerized measurements (Sela et al., 1990) by a semi-automatic morphometric system of Galai Ltd (http://www.galai.co.il.), known as the Wscan Array Image Analyzer, version 2.23, utilizing information acquired with a digital camera directly connected to the microscope. The digital photography using a DP11 Olympus camera was followed by analysis using the Sigma Scan Pro program.

Each microscopic field (of the cell-culture wells) was photographed twice (sequentially, with the plate fixed in place), once with a mechanical camera (Olympus model OM-2) and once with a digital camera (Olympus model DP-11), having a resolution of 1024 × 1080 pixels. Both cameras were microscope-mounted, in such a way that they both saw exactly the same field frame at the very same time and under the same lighting conditions. The films exposed via the mechanical camera were developed, and prints were prepared on glossy black-and-white Kodak paper. The digital photos were acquired with a DP-software program, so that they could be transferred as JPEG files to the Galai Wscan PC-based system. In order to reduce the loss of information, the JPEG files were not opened unnecessarily.

For each field of view of each NGF concentration, 12 pictures were analyzed, following acquisition by both cameras. The following guidelines were used: (a) A neurite was defined as a cell-extension whose length was at least twice the diameter of the cell; (b) At least 100 cells had to be analyzed for each NGF concentration; (c) Only cells within the field frame of the camera were to be analyzed; (d) Neurites would not be included in the count, unless they were completely within the field frame.

Method # 1. Manual analysis: manual analysis of neurites on printed photographs is time consuming (Ronn et al., 2000), and was performed by marking the neurites and the cells from which they sprouted, with color markers. All pictures were taken at × 250 magnification (on a micrometer scale), using an Olympus OM-2 camera and DP-10 digital camera simultaneously attached to an Olympus inverted light microscope (Hamburg, Germany). The film type used for the mechanical camera was 36-mm 125-ASA Ilford FP-4 black-and-white. Cell and its neurites were marked with the same color, to differentiate them from the surrounding cells. The length of each neurite, as well as the diameter of its neural cell, was then measured manually by using a ruler (in mm); and then the number of cells in each field were counted, and the percentage of the cells with neurites in each field was calculated.

Method # 2: Galai C-3 system: the negative films of the manually-analyzed prints were analyzed by a Galai C-3 morphometry system, after being placed above a light source (Illumitran 3 model, made by Bowens, England). Then the pictures were transferred to the computerized system via a video color CCD camera (Model M-852, made by Micro-Technica, Japan) through a 55-mm lens (55-mm Micro Nikkor, Nikon, Japan). Digitalization of the picture was performed by the PC, and detected by the computer's mouse. Analysis was performed on a Sony Trinitron screen, having a resolution of 512 × 512 pixels, with 256 gray levels. The analysis included object marking and studying by computerized morphometric analysis, in order to measure the radius of the cell and the length of the neurites.

Method # 3: D-Scan method: digital acquisition and computerized analysis: Photographs were taken by using the digital camera (DP11, Olympus, Hamburg Germany) with a resolution of 1024 × 1080 pixels. The digitized pictures were transferred as JPEG files from the DP-soft program (which was used to acquire them from the digital camera) to the Galai Wscan Array system (Galai Electro-optic Laboratories for Research and Diagnosis Ltd, Migdal-Haemek, Israel). For each picture a primary computerized editing was performed, in order to distinguish between the cell bodies, neurites, and background, so that an optimized contrast picture could be obtained at any time frame, with great care given to preserving thin neurites. In addition mode, a macro route was designed so that each picture could be handled in a pre-determined sequence of events. The editing process was aimed to separating the cell bodies, neurites, and background, so that a similar contrast picture could be obtained in each instance. Then, using the computer's mouse, we designated with the mouse the areas to be morphometrically evaluated, and the radii of the cells, as well as the lengths of the neurites.

2.7. Statistical evaluation

Data were accumulated three times from about 110 pictures, and were fed into a database designed by an MS Excel program. Unless otherwise indicated, one hundred cells were evaluated, the mean of PRC and E and SEM of three independent experiments were calculated. SEM values were found lower than 5% of the mean values. ANOVA test was performed separately for each NGF concentration, in order to evaluate the significance of the difference between

the three methods. Paired Student's *t* test was performed in order to evaluate the difference between the groups. In addition, calculation of logit regression curves and their statistical analyses were performed as previously described (Katzir et al., 2002).

3. Results

3.1. The digital method is advantageous over the manual one for measuring the NGF neurotropic effect in the PC12-6.24-I bioassay

Fig. 1b shows trkA receptor-overexpressing PC12 cells that have grown neurites under treatment with 50 ng/ml NGF for 7 days while Fig. 1a represents the untreated control cells. For each NGF concentration, and for all three methods, we counted the number of cells in all fields of view that were photographed, as well as the number of neurite-bearing (differentiated) cells. Fig. 1c is an estimation of the logit PRC regression model from NGF concentrations by the three methods evaluated. One can see that the percentage of differentiating cells is growing in a dose-response manner within a concentration range of 0.1–10.0 ng/ml, as determined by all three methods. Statistical evaluation of these three lines for parallelism (chi-square test) demonstrated that there was no significant difference between the manual and the D-scan methods ($p = 0.41$), while there was a statistical difference between the manual and the C-3 method ($p = 0.01$). We also recorded the time (in minutes) that it randomly took to analyze 10 pictures by each of the three methods. We compared mean values, as with time and experience, the evaluation-time becomes shorter. While the manual method required about 50 min to evaluate 10 pictures, the corresponding time for both computerized methods was about 30 min, with no significant difference between the two computerized methods. Our results thus suggest that both computerized methods are time-saving, and that the D-Scan method yields more reliable values for neuronal differentiation than the C-3 method does.

In conclusion, digital photography and computerized analysis (the D-Scan method) provide similar data as the manual method, yet, the D-Scan method is more convenient due to the faster acquisition of data and accurate analysis.

3.2. 7S m-NGF induces neurite outgrowth from wild-type PC12, but not from PC12-6.24-I

PC12wt cells initiate neurite outgrowth upon treatment with 2.5S β-NGF or with its precursor 7S NGF (Shao et al., 1993), therefore, we treated both cell lines with 7S NGF. While PC12wt responded as expected by neurite outgrowth

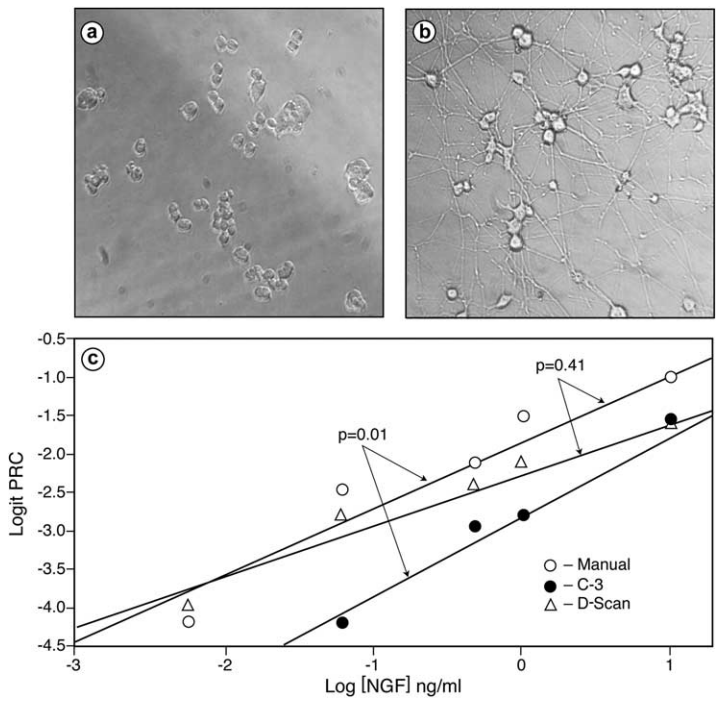

Fig. 1. Comparison of different methods for evaluation of NGF-neurotropic effect in a bioassay using trkA-overexpressing PC12 cells (PC12-6.24-I). Microscopic photographs (× 100) of PC12-6.24-I cells cultured for 7 days without (a); or with 50 ng/ml NGF (b); logit regression of dose response effects, estimated by PRC (c) using three different methods. The statistical significance is indicated by p values.

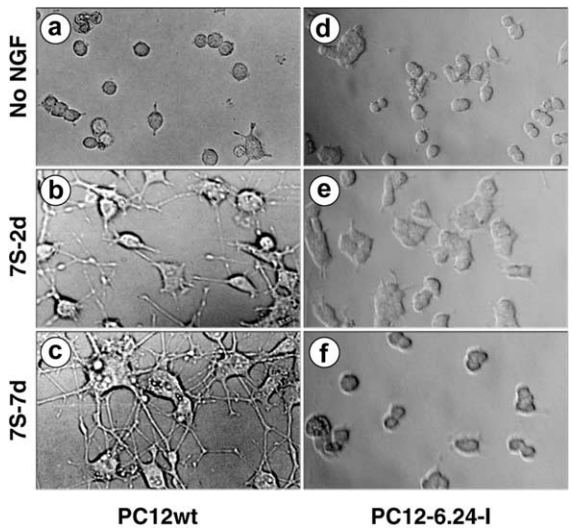

Fig. 2. Lack of neurite outgrowth in trkA-overexpressing PC12 cells (PC12-6.24-I) compared to wild type PC12 cells (PC12wt) upon 7S NGF treatment. a and d—untreated cells (no NGF); b, e and c, f—cell treated with 7S NGF 50 ng/ml for 2 days (2 d) or 7 days (7 d), respectively, (× 250).

(Fig. 2), the PC12-6.24-I cells did not respond to 7S NGF (Fig. 2). These results indicate that transfected rh-trkA receptors in PC12-6.24-I are unable to respond biologically to 7S NGF. Quantification of 7S NGF-induced neurite outgrowth was evaluated from dose-response curves at 2 and 7 days of treatment (Fig. 3). Analyzing logit PRC yielded a significant effect of 7S NGF concentration ($p < 0.001$), and also a significant difference between 2 and 7 days ($p < 0.005$), also a significant interaction between NGF concentration and number of days of treatment ($p = 0.044$) was found. For the variable E, a significant effect for 7S NGF concentration was observed ($p < 0.0001$). The difference between the effects at days 2 and 7 did not reach statistical significance ($p = 0.076$), yet, the interaction between days of treatment and NGF concentration was significant ($p = 0.025$).

3.3. 2.5S rh-NGF is effective in the PC12-6.24-I bioassay

2.5S rhNGF was evaluated in the PC12-6.24-I bioassay (Fig. 4). Cells treated with this NGF isoform showed an extensive neurite outgrowth (Fig. 4b) when compared with the control untreated cells (Fig. 4a). In this experiment, at least 90 cell were scored for each concentration, and the SD's values were lower than 5% of the mean values. A quantitative analysis of the neurite outgrowth effect is presented in Fig. 4c and d, analyzed by both manual and digital methods. No statistical difference ($p > 0.05$) between the data evaluated by manual and digital methods was observed (Student's paired t test). Both methods provided similar values and an EC_{50} of 0.4 ± 0.04 ng/ml for PRC and of 0.2 ± 0.05 ng/ml for E, indicating a higher activity compared with that of 2.5S mNGF (Table 1).

3.4. Snake NGF evaluation in PC12-6.24-I bioassay

The quantitative neurotropic effect of snake venom NGF is presented in Fig. 5. Since in the past these NGFs were evaluated semi-quantitatively on PC12wt only, we compared their neurotropic effect between PC12wt and the PC12-6.24-I clone. This comparison was performed in optimal conditions of the bioassay, i.e. 7 days for PC12wt, and 2 days for PC12-6.24-I (Katzir et al., 2002). The snake venom NGFs evaluated were definitely not toxic to either clone, and progressively induced neurite outgrowth similar to that of mouse NGF. Dose-response curves were generated (Fig. 5), and the EC_{50} values are presented in Table 1. Both dose-response curves and EC_{50} values indicate the high potency of mouse NGF compared with the lower potency of snake's NGF. Also, it is evident from the data obtained with both clones that

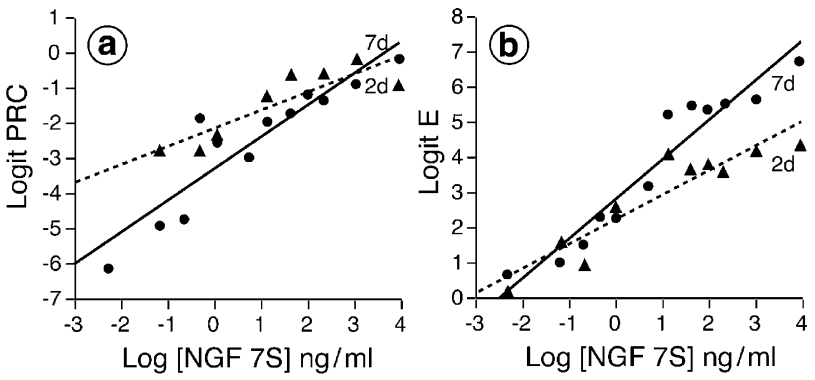

Fig. 3. Quantitative dose-response curves of neurite outgrowth induced by 7S NGF in wild type PC12 cells (PC12wt). Triangles represent 2 days (2 d) and circles represent 7 days (7 d) of treatment. PRC = percentage of responsive cells (a); E = the ratio between neurite outgrowth and cell diameter (b).

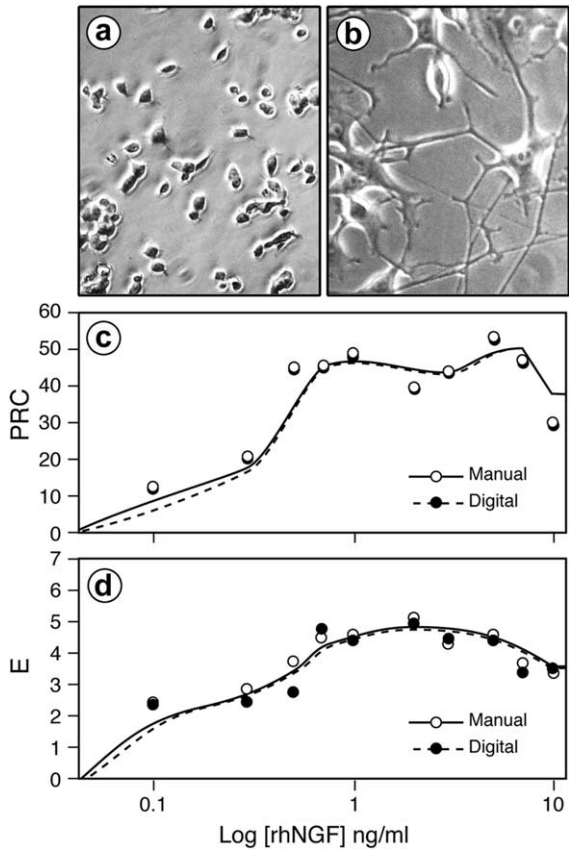

Fig. 4. Activity of recombinant human NGF in the bioassay using trkA-overexpressing PC12 cells (PC12-6.24-I). PC12-6.24-I without (a) and after 2 days treatment with (b) 20 ng/ml rhNGF (× 250). Dose-response curves of PRC (c) and E (d) and rhNGF concentration in the ng/ml range were prepared by the manual (open circle) and digital (solid circle) methods.

nnkNGF was more potent than vrrNGF. By comparing the efficacy of snake venom's NGF to that of mouse NGF, considering the SEMs, we can conclude that their efficacy was similar to that of mouse NGF.

Table 1
EC50 values of NGFs from different biological sources, evaluated on PC12wt and PC12-6.24-I bioassay

NGF	PC12-6.24-I		PC12wt	
	PRC	E	PRC	E
rhNGF	0.4 ± 0.04	0.2 ± 0.05	0.8 ± 0.03	0.8 ± 0.02
mNGF	1.8 ± 0.04	0.5 ± 0.03	0.8 ± 0.04	0.7 ± 0.03
nnkNGF	10.0 ± 0.4	6.5 ± 0.31	9.6 ± 0.41	7.4 ± 0.37
vrrNGF	60.0 ± 2.0	10.0 ± 3.1	78.0 ± 3.8	30.0 ± 1.6

The neurotropic effect of NGFs was evaluated on PC12wt at 7 days and on PC12-6.24-I at 2 days. EC_{50} (ng/ml) values represent mean ± SEM.

An additional interesting finding was the stability of the nnkNGF-induced neurotropic effect, as compared with that of the mouse NGF-induced one (Fig. 6). In order to maintain the neurite outgrowth effect, continuous exposure of the cells to mNGF is obligatory, which is achieved by replacing the medium with fresh NGF every 2 days (Fig. 6a). If fresh mNGF is not added at this frequency to the medium, the neurite outgrowth deteriorates after 7 days, as visualized in Fig. 6b, most probably due to NGF degradation in the medium. Upon exposure of the culture to nnkNGF (but not to vrrNGF), a prolonged, stable, neurite outgrowth effect is observed, both with fresh NGF (Fig. 6c) and without the addition of fresh NGF (Fig. 6d). We assume that this effect represent either a higher $t_{1/2}$ of nnkNGF compared to mouse NGF and vrrNGF or a more stable activation of trkA receptors.

4. Discussion

This study utilizes a novel bioassay to NGF based on PC12 cells overexpressing the rhtrkA NGF-receptor, and adjust it to be suitable for measuring the neurotropic effect of NGFs from snake venom. In addition, this bioassay showed a high potency for rhNGF and a differential response between mouse 2.5S and 7S NGF; i.e. whereas 2.5S NGF was neurotrophic, 7S NGF was non-active. The finding that rhNGF demonstrated the highest potency among the various NGFs was expected, considering the fact that this bioassay, in contrast to the PC12wt bioassay, mainly reflects the NGF effect on the human trkA transfected receptors in the trkA transfected cells (Katzir et al., 2002). By comparing the potency of the various investigated NGFs, the quantitative analysis clearly indicate the following order of potency: rhNGF > mNGF > nnkNGF > vrrNGF. The crystal structure of rhNGF in complex with the ligand-binding domain of the rhtrkA receptor had shown that the ligand-receptor interface consisted of two patches, one involved with core region of the homodimeric NGF molecule, and the other involved with the N-terminal region of NGF (Wiesmann et al., 1999). When the amino acid sequence of rhNGF was compared with those of other NGFs, replacements of four, eight, and nine amino acids were found in mNGF, nnkNGF, and vrrNGF, respectively, among 33 residues of these patches interacting directly with the trkA receptor. Therefore, it is conceivable that the difference in the results of quantitative bioassay using PC12 cells, reflects the difference in the affinities to trkA receptor due to the amino acid replacements of these NGFs.

In the past, when the biological activities of snake venom's NGF were assayed utilizing PC12wt, all snake venom NGFs demonstrated the same biological activity as mNGF (Hayashi et al., 1996). This apparent discrepancy may be explained by the semi-quantitative property of the PC12wt bioassay, which utilizes a low level of rat's trkA

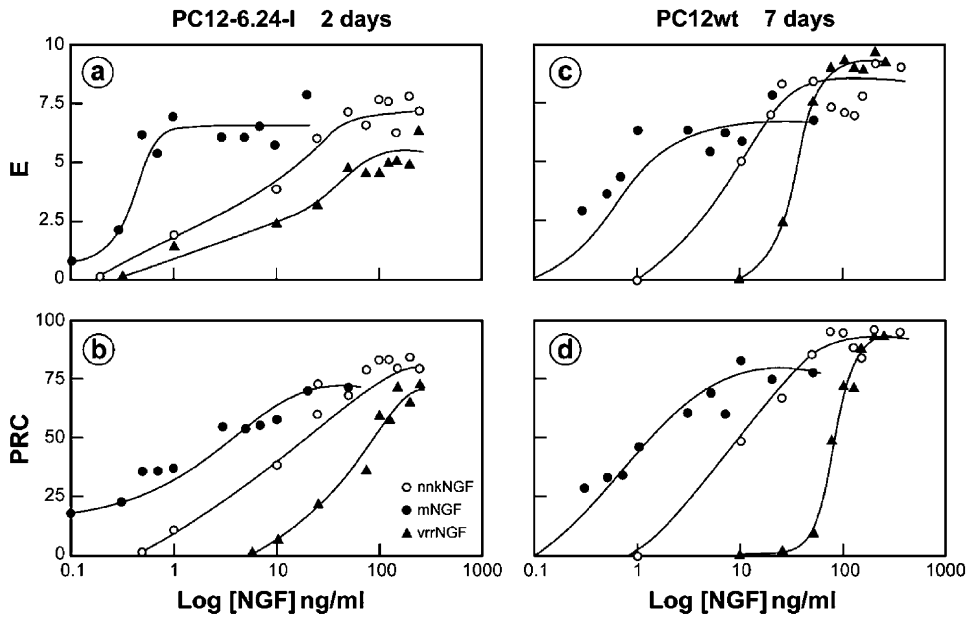

Fig. 5. Dose-response-curves of snake venom's NGF and mouse NGF compared in the wild type (PC12wt) and trkA overexpressing (PC12 6.24-I) PC12 cells bioassay. NGFs from *Naja naja kaouthia* (nnkNGF) and *Vipera russelli russelli* (vrrNGF), and mouse β-NGF (mNGF) were tested on two PC12 clones, at day 2 for PC12 overexpressing trkA (a,b) or at day 7 for the wild-type PC12 (c,d). Neurite outgrowth response was evaluated in terms of PRC (b,d) and E (a,c).

receptors. The present study is the first evaluating, in a precise quantitative manner, the neurotrophic activity of the snake venom NGF, by using human trkA receptors. At the level of human trkA receptor, the potency of NGF was different. Indeed, such a different biological response was observed in the past when the snake venom's NGF were tested on the chicken DRG. For example, cobra NGF demonstrated a lower neurotropic activity towards DRG-cells as compared to mNGF (Server et al., 1976). With respect to the increasing length of the neurites (Hayashi et al., 1996), the length of neurites evaluated in the present study by the parameter E represent a good measure of the extent of NGF-induced neurite outgrowth. However, this parameter reflects mainly the neurite elongation process, but not the overall response of the neuronal population. In the present study we investigated

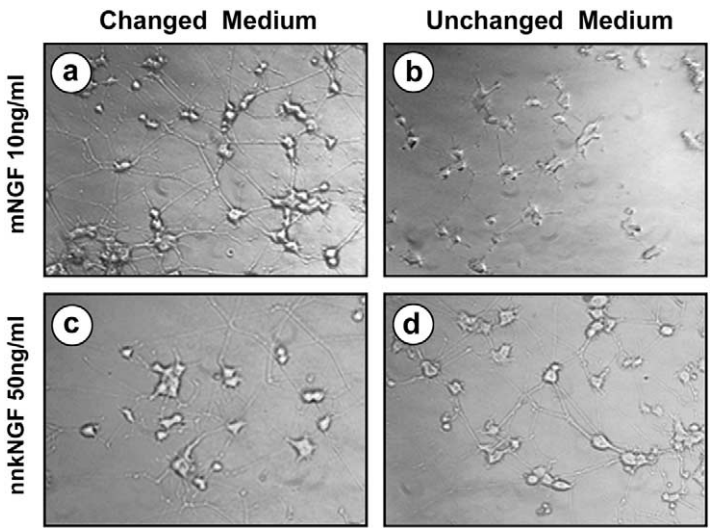

Fig. 6. Stability of neurotropic effect induced by NGF from *Naja naja kaouthia* (nnkNGF) in contrast to mouse β-NGF (mNGF). PC12-6.24-I cells were treated with mNGF or with nnkNGF. The medium was changed every 2 days (a, c) or left unchanged for 7 days (b, d).

an additional parameter PRC, which reflected the percentage of the neuronal population responding by neurite outgrowth. Therefore, by comparing both parameters obtained from the same measurements, we can conclude better the characteristics of snake venom's NGF. Table 1 clearly indicates the same tendency for both PRC and E values, independent of the bioassay or type of NGF tested. When the value of PRC was higher, the E value was higher too.

These two parameters (E and PRC) are both needed, since each of them may reflect a different biological response: E—polymerization of the neurite cytoskeleton, the rate-limiting factor for neurite outgrowth, and PRC—the reflection of the heterogeneity of the neuronal population in the cell cycle. Only cells in the G_0 of the cycle can initiate the differentiation process of neurite outgrowth (Rudkin et al., 1989). Another important aspect of the present study is the similar efficacy of all NGFs tested. This pharmacological finding further emphasizes the strength of the present bioassay using PC12-6.24-I. The phylogenetic tree of snake venom NGFs clearly indicates both the evolutionary conservation and divergence of snake venom's NGF with respect to mammalian NGFs. Snake venom's NGF, represents a rich, versatile family of trkA receptor agonists that may be helpful in toxicological and pharmaceutical research. Therefore it is desirable to isolate and identify other NGFs, from the different snake venoms and other biological sources. Indeed, we have attempted in our laboratory to use the present bioassay using PC12-6.24-I, to isolate NGFs from venoms of *Naja naja mozambica*, *Bungarus multicinctus*, and *Vipera palestinae*. Several HPLC fractions of these snakes venom's demonstrated NGF-like activity (data not shown). PC12-6.24-I bioassay will provide a novel, accurate and sensitive tool to isolate and characterize the above venom's NGF. The introduction of our digital method for evaluation the morphology of the cells (measuring their area) and neurites (measuring their length) in conjunction with the computerized method for calculating and analyzing the measured data will contribute to the further progress of research on NGFs from venoms.

In summary, the present study describes a novel digital-computerized method for evaluation of NGF-induced neurite outgrowth. By the use of this bioassay we found that cobra and vipera venom's NGF represent natural agonists for human trkA receptor of lower potency, but of similar efficacy when compared with mammalian NGFs. These venom's NGF may be important pharmacological tools to investigate human trkA receptor under physiological and disease conditions.

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