

Nerve growth factor is preformed in and activates human peripheral blood eosinophils

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Background: Recent studies have shown that nerve growth factor (NGF) is produced by and can act on several immune-inflammatory cells.

Objectives: The objective of this study was to study the effects of NGF on human peripheral blood eosinophils and assess whether these cells produce and store NGF.

Methods: Eosinophils were purified by negative immunoselection (magnetic cell sorting systems, purity 98% to 100%) from 13 subjects (9 to 26 years old) with mild blood eosinophilia, mainly of allergic origin. Eosinophils were incubated with NGF (50 to 1000 ng/mL), and supernatants were collected for measurement of eosinophil peroxidase (EPO, 20 minutes, colorimetric enzymatic assay) and IL-6 (12 hours, ELISA). Eosinophil viability was evaluated by Trypan blue test (days 2, 3, and 4). NGF content in freshly isolated eosinophils, after ultrasound disruption, was determined with a 2-site immunoenzymatic assay. The presence of mRNA for NGF was evaluated by reverse transcription PCR.

Results: NGF caused EPO release (highly significant at 1000 ng/mL NGF). IL-6 release from eosinophils was not higher than IL-6 spontaneously released into culture medium alone. NGF did not significantly affect the number of viable eosinophils. NGF was found in the eosinophil sonicates (1.5 to 17.8 pg/mL per 10⁶ cells). Similarly, mRNA for NGF was detected by reverse transcription PCR in the freshly isolated eosinophils.

Conclusions: NGF activates human peripheral blood eosinophils from subjects with mild eosinophilia to selectively release inflammatory mediators. Eosinophils store and produce NGF. Therefore the capability of NGF to induce a secretory response and its production and storage by circulating human eosinophils suggest a possible role for NGF in conditions associated with eosinophilia, including allergic disease. (*J Allergy Clin Immunol* 1998;102:454-60.)

Key words: Eosinophil peroxidase, IL-6, nerve growth factor, allergic diseases

Nerve growth factor (NGF) is a neurotrophic polypeptide essential for the development, survival, and normal function of neurons.^{1,2} Several data recently published indicate that NGF can also have a role in allergic and inflammatory diseases.³ In fact, NGF causes hypertrophy of mast cells in newborn mice⁴ and in vitro degranulation of mast cells and mediator release.⁵ Moreover, NGF shifts the mucosal phenotype of IL-3-dependent, bone marrow-derived mast cells into the connective tissue one.⁶ The prerequisite for the direct effect of NGF on mast cells is the presence of a receptor for NGF on their membranes (Trk-A).⁷⁻⁹ In addition, NGF is a preformed mediator in human and rodent mast cells, which are a source for this mediator.⁸⁻¹⁰

The connection between mast cells and NGF recently led researchers to investigate serum levels of NGF in patients with allergies who have asthma, rhinoconjunctivitis, and urticaria-angioedema. In these patients NGF levels were found to be enhanced when compared with control subjects.¹¹ High levels of NGF were recently demonstrated in the serum of patients with vernal keratoconjunctivitis, and a positive correlation was found between NGF levels and the number of mast cells in biopsy specimens of tarsal and bulbar conjunctiva of these patients.¹² In addition, human CD4⁺ T-cell clones express high-affinity NGF receptors.¹³ They also produce and release this neurotrophin, with a preferential participation of T_{H2}-type clones after mitogen stimulation.¹³

Eosinophils, as well as mast cells and T_{H2} cells, play a central role in the pathogenesis of allergic diseases. Activation of mast cells by IgE-mediated mechanisms causes cell degranulation, consequent release of various mediators, and recruitment of inflammatory cells, with an activated phenotype, to target tissues. The eosinophils are the predominant inflammatory cells of the late phase and chronic development of allergic inflammation. Activated eosinophils secrete many inflammatory and proinflammatory mediators, including basic proteins (major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase), phospholipid metabolites, and several cytokines.¹⁴⁻¹⁶ Very few data are available on the effect of NGF on human eosinophils,^{17,18} and no information is available on whether these cells are a

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Abbreviations used

EPO: Eosinophil peroxidase

NGF: Nerve growth factor

source of and store this neurotrophin. Indeed, we have recently shown that the eosinophilic inflammatory infiltrate from biopsy specimens of patients with vernal keratoconjunctivitis shows an enhanced expression of NGF high-affinity receptor and reacts with anti-NGF antibodies,¹⁹ indicating the presence of NGF.

We have hypothesized, therefore, that NGF could affect eosinophils, and it may also be contained in these cells as a preformed mediator. Therefore the purpose of this study was to investigate the possible effect of NGF on peripheral blood eosinophils from volunteers with mild eosinophilia of mainly allergic origin and to assess whether human circulating eosinophils store and produce NGF.

METHODS

Subjects

Thirteen subjects (mean age, 18 years; range, 9 to 26 years) were studied. All subjects had mild blood eosinophilia (range, 4% to 10%). The majority of subjects had signs and symptoms of allergic disease: 5 patients had vernal keratoconjunctivitis, 3 patients had allergic rhinitis, 2 patients had allergic conjunctivitis, and 1 patient had mild bronchial asthma. Two subjects were asymptomatic, and the cause for their eosinophilia could not be attributed to any specific morbid condition by common examination but to a family history of atopy. No subject received any systemic or local treatment for 1 week before blood withdrawal. Informed consent was given by all subjects or by parents for those under age 18 years, in accordance with the Helsinki declaration.

Isolation of eosinophils

Eosinophils were purified from the peripheral blood as previously described.¹⁵ Briefly, venous blood (100 to 150 mL) was collected in heparinized syringes and left to sediment in 6% dextran (Sigma Chemicals, St Louis, Mo). Leukocytes were centrifuged on Ficoll-Paque (density 1.077) (Sigma Chemicals) for 25 minutes at 700g at 4°C. Neutrophils in the granulocyte-enriched pellet were tagged with micromagnetic beads to anti-CD16 antibodies, lymphocytes with anti-CD3 antibodies, and mononuclear cells with anti-CD14 antibodies (Miltenyi, Biotec GmbH, Bergisch Gladbach, Germany). The tagged cells were then eliminated by passing them through a magnetic field (MACS). Eosinophils were collected at a purity of 98% to 100% as assessed by Kimura staining and at a viability of more than 99% as assessed by Trypan blue staining (Sigma Chemicals). Isolated, purified eosinophils were incubated with 80 ng/mL recombinant human GM-CSF (Genzyme, Cambridge, Mass) to validate their ability to survive under established optimal conditions. In these experiments, 85% $\pm$ 2.8% eosinophils were alive on the fourth day of incubation ($n = 4$).

Culture of eosinophils

Freshly purified eosinophils were resuspended in culture medium (RPMI-1640 Biological Industries, Beith Haemek, Israel) supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, 2 mmol/L L-glutamine, 1 mmol/L HEPES, and 5% FCS (Biological Industries), enriched medium, at a concentration of 1×10^6 cells/mL. One hundred microliters of the suspension was placed in each well of a 96-well microtiter plate (NUNC, Roskilde, Den-

mark). NGF, prepared from mouse salivary glands by the method described elsewhere,²⁰ was dissolved in enriched medium at a concentration of 1 mg/mL and stored in aliquots at -80°C . Before each experiment, NGF was thawed at room temperature, diluted, and then added to the wells at final concentrations of 50, 100, 500 and 1000 ng/mL. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 (20 minutes for EPO, 12 hours for IL-6, and up to 4 days for viability determination, respectively).

NGF biologic activity was evaluated by in vitro neurite outgrowth bioassay, with dorsal root ganglia from 8-day-old chick embryos. Under this condition, NGF concentrations of 5 to 10 ng/mL were able to stimulate neurite growth after 12 hours.²¹

Assessment of viability

Triplicates of wells containing eosinophils incubated with either enriched medium or the 4 different concentrations of NGF were incubated for 2, 3, and 4 days for assessment of viability as follows. At 2, 3, and 4 days, 20 μ L was recovered from each well after resuspension of the cells, and the viability was assessed by the Trypan blue exclusion test. The percentage of surviving cells was calculated according to the following formula: Viability = Number of viable eosinophils per well $\times$ 100/initial number of seeded viable eosinophils.

Consequently, 20 μ L of medium containing similar concentrations of NGF was added to the wells to restore the initial volume of the wells. To evaluate possible cytotoxic effects of NGF, eosinophils isolated from 5 patients were cultured with the above NGF concentrations, and their viability was assessed after 24 hours of incubation.

Eosinophil peroxidase and IL-6 determination

For eosinophil peroxidase (EPO) determination, eosinophil cultures in quadruplicate were incubated for 20 minutes with different concentrations of NGF or medium alone. At this time, supernatants were collected by centrifugation (5 minutes, 120g) and stored at -80°C until assessed. EPO was determined by using as a substrate *O*-phenylenediamine dihydrochloride (Sigma Chemicals) by a colorimetric assay as previously described.²²

IL-6 was quantified by a commercial ELISA kit specific for human IL-6 (Quantikine, R&D Systems, Minneapolis, Minn). The lower limit of assay sensitivity for IL-6 was 0.7 pg/mL. Results are expressed as pg/mL IL-6 generated by 1×10^5 eosinophils.

Determination of NGF content in eosinophils

To determine NGF content in eosinophils, freshly isolated eosinophil sonicates (1×10^6 /mL in DMEM) were prepared by continuous sonication for 1 minute in ice (Heat Systems Ultrasonics, 50% duty cycle, output 5). NGF levels were measured in the eosinophil sonicates by using a highly sensitive 2-site immunoenzymatic assay with anti- β -NGF galactosidase (Boehringer, Mannheim), which is capable of detecting as low as 5 pg/mL NGF.²³ Data were expressed as picograms per milliliters. All assays were performed in triplicate.

RNA isolation and reverse transcription PCR amplification

Total RNA was extracted from 3 to 5×10^6 (100% pure) freshly eosinophils or from human foreskin fibroblasts cell line HS68 (ATCC, Rockville, Md) by using the commercial reagent (TRI-REAGENT) based on the acid guanidinium-thiocyanate RNA extraction technique.²⁴ First-strand cDNA synthesis reaction was catalyzed by Super Script II Rnase H-Reverse Transcriptase and oligo (dT)₁₂₋₁₈ primer according to the manufacturer's instructions. The generated complementary DNA was amplified in a total volume of 25 μ L with 1.25 U of Taq DNA polymerase and 0.2

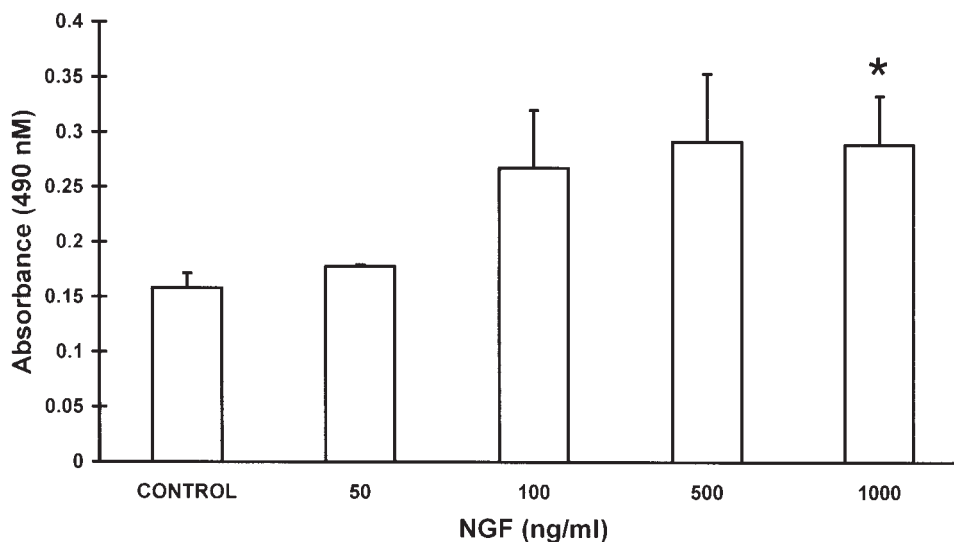


FIG 1. EPO release from eosinophils cultured with NGF. EPO levels were assessed in supernatants of isolated eosinophils cultured for 20 minutes with different NGF concentrations by enzymatic colorimetric assay. Data are mean $\pm$ SEM of experiments performed with eosinophils isolated from 13 subjects with mild eosinophilia. Assays were performed in quadruplicate. * $P = .0025$ as determined by Friedman non-parametric repeated-measures ANOVA.

mmol/L dNTP mixture, 0.4 μ mol/L of NGF primers, and 10% glycerol as a specificity enhancement. The primer sequences for NGF were 5' CAG GAC TCA CAG GAG CAA ACG and 3' TCG GAA GGA CGA CTC GTC TGT, generating a fragment of 370 bp. The primer sequences for G3PDH, used as a control to test the efficiency of cDNA synthesis, were 5' ACC ACA GTC CAT GCC ATC ACT GCC and 3' CAT GTG GGC CAT GAG GTC CAC CAC, generating a fragment of 468 bp. The specificity of the primers was confirmed by the manufacturer.

NGF mRNA has been shown to be present in human foreskin fibroblasts.²⁵ RNA of these fibroblasts was consequently used as a positive control. The thermocycler PTC-100 (MJ Research Inc, Watertown, Mass) was used for the PCR amplifications with the following settings: 35 cycles at 95°C for 1 minute, 57°C for 1 minute, and 78°C for 2 minutes. Amplified products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, and photographed under UV light.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical analysis was performed with the unpaired Student *t* test or the nonparametric repeated-measures analysis of variance where appropriate. A value of $P < .05$ was considered significant.

RESULTS

Effect of NGF on EPO release

Twenty-minute incubation of eosinophils with various concentrations of NGF caused a dose-dependent EPO release compared with incubation of eosinophils in medium alone (Fig 1). EPO release was significantly higher when the effect of 1000 ng/mL NGF was compared with that of medium alone (0.158 ± 0.013 OD [in medium] compared with 0.289 ± 0.044 OD [with 1000 ng/mL NGF]) ($P = .0025$ as determined by nonparametric repeated-measures ANOVA).

Effect of NGF on eosinophil survival

The viability of eosinophils incubated with different concentrations of NGF for 24 hours did not differ significantly from that of eosinophils incubated in medium alone (55.20% for 1000 ng/mL NGF versus 57.02% for medium alone, $P = .07$, $n = 5$) (Fig 2, A).

After 2 days, the viability of eosinophils cultured in medium alone was $31.90\% \pm 7.03\%$ (Fig 2, B). Culturing eosinophils with different NGF concentrations did not significantly change their viability compared with medium alone ($P = .1$). However, a lower but not significant viability was observed when eosinophils were cultured with 100 ng/mL NGF ($21.90\% \pm 4.19\%$ viable eosinophils).

After 4 days of culture, no significant differences in eosinophil viability were noted between medium alone and the different NGF concentrations (Fig 2, B). Viability values at day 4 ranged between $4.64\% \pm 1.30\%$ (in medium) and $5.62\% \pm 1.46\%$ (with 500 ng/mL NGF).

Effect of NGF on IL-6 release

Eosinophils incubated for 12 hours in medium alone spontaneously released IL-6. This basal release was not significantly affected by any of the different NGF concentrations. IL-6 levels in the supernatants of eosinophils cultured with NGF ranged between 1.82 ± 0.38 pg/mL (50 ng/mL NGF) and 1.84 ± 0.42 pg/mL (1000 ng/mL NGF). Eosinophils incubated in medium alone released 2.12 ± 0.64 pg/mL of IL-6 (Fig 3).

NGF content in eosinophils sonicates

Freshly isolated eosinophils from 7 subjects were disrupted by sonication for the determination of NGF cell

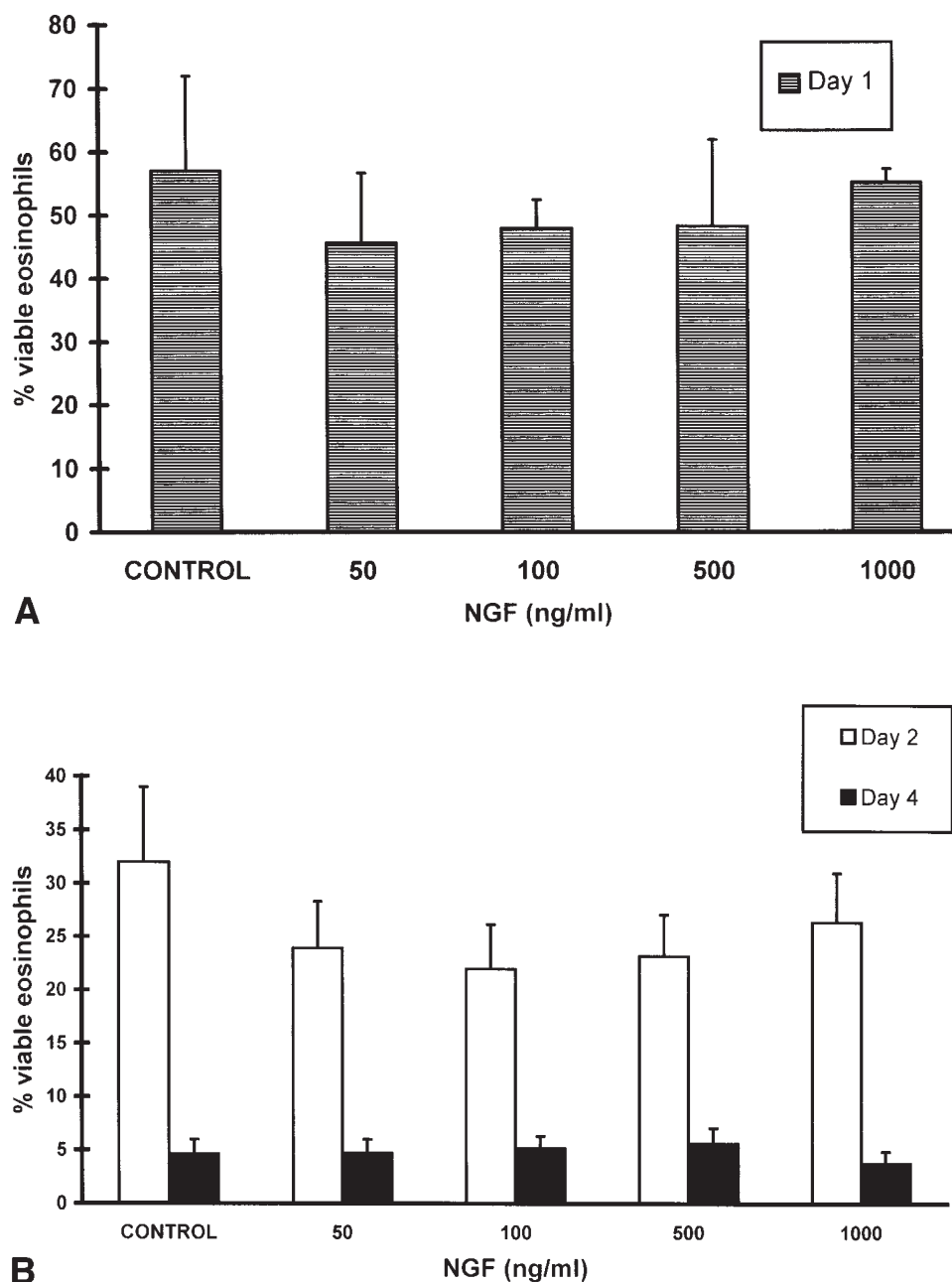


FIG 2. Viability of eosinophils cultured with different NGF concentrations. Viability was assessed by Trypan blue exclusion test after 24 hours in 5 subjects (**A**) and after 2 days and 4 days in 13 subjects (**B**). Data are mean $\pm$ SEM of experiments performed with eosinophils isolated from subjects with mild eosinophilia. Assays were performed in triplicate.

content. NGF was found in the sonicates of all subjects, ranging from 0.64 to 17.8 pg/mL per 10^6 cells (average, 4.02 ± 2.32 pg/mL per 10^6 cells).

Expression of mRNA for NGF in eosinophils

To assess whether NGF present in circulating eosinophils as a preformed mediator is produced by these cells, reverse

transcription PCR was used to investigate the presence of mRNA for NGF in eosinophils isolated from different volunteers. As shown in Fig 4, freshly isolated eosinophils displayed a transcript for NGF as the human foreskin fibroblasts, used as positive control.²⁵ Both eosinophils and fibroblasts expressed mRNA for G3PDH. These data confirm the ability of eosinophils to produce NGF (Fig 4).

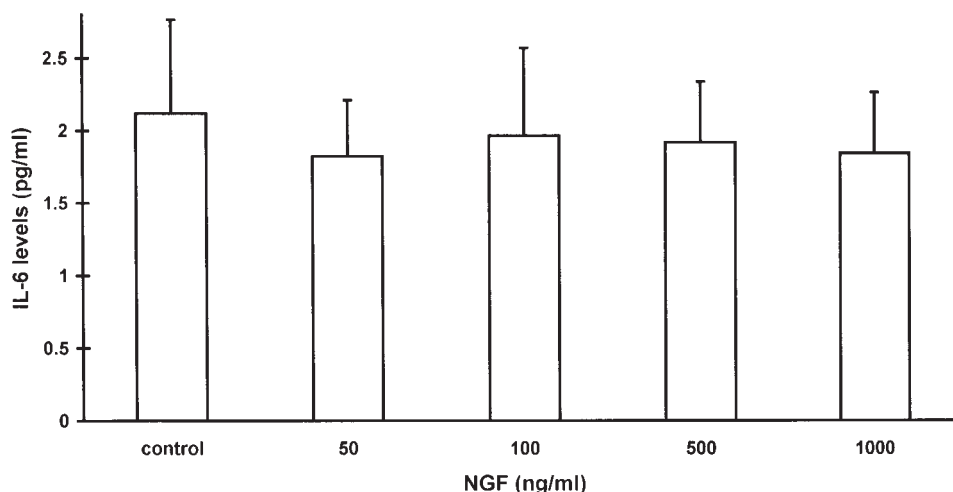


FIG 3. IL-6 release from eosinophils cultured with NGF. IL-6 levels were assessed in supernatants of isolated eosinophils cultured for 12 hours with different NGF concentrations by ELISA kit. Data are mean $\pm$ SEM of experiments performed with eosinophils isolated from 13 subjects with mild eosinophilia. Assays were performed in triplicate.

DISCUSSION

Attention and interest have recently focused on the role NGF plays in allergic and immunologic diseases.¹⁰⁻¹² Several studies indicate that NGF can affect various immune and inflammatory cells through specific receptors present on their membranes.⁷⁻⁹ On the other hand, NGF is also produced and stored by immune cells such as mast cells and CD4⁺ cells.^{8-10,13}

In this report we show that NGF can induce circulating human eosinophils to release EPO, an important proinflammatory mediator. This release is not a result of a cytotoxic effect of NGF on the cells because no significant increase in the mortality rate of eosinophils was observed after 24 hours of culturing. In addition, the lack of a significant IL-6 release, which is a granule-associated mediator,¹⁶ after 12-hour culturing of eosinophils with NGF further strengthens the fact that NGF is not cytotoxic to eosinophils.

The fact that exogenous NGF is able to activate eosinophils and to stimulate the release of a toxic mediator such as EPO from these cells suggests that NGF may exacerbate tissue damage during allergic reaction. However, the possibility that NGF through its neuroimmune regulation might be involved in reparative events cannot be excluded. Our data indicate that in contrast to EPO release, NGF does not influence IL-6 release from eosinophils. In another study, NGF has been shown to have an inhibitory effect on LTC₄ release.¹⁷ It has been suggested that releasability of human eosinophils is a selective phenomenon,²⁶ and it is therefore conceivable to speculate that EPO, IL-6, and the arachidonic acid metabolite LTC₄ have different mechanisms of release and undergo different modulatory control.

IL-6 is a key cytokine in inflammatory responses and an important regulatory factor in allergic inflammation.

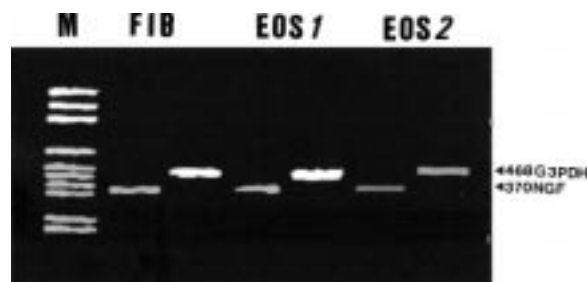


FIG 4. Reverse transcription PCR analysis of NGF and 3GPDH in freshly isolated human eosinophils. Total RNA was used as template for PCR with primer specific for NGF mRNA (see Methods section). PCR products of eosinophils from 2 different subjects (EOS1 and EOS2) and from human foreskin fibroblast cell line (FIB) were analyzed by electrophoresis in a 1.8% agarose gel. Amplified DNA was stained with ethidium bromide and photographed. Positions of NGF- and 3GPDH-specific PCR products are indicated by arrowheads.

In fact, it participates in IL-4-dependent IgE synthesis from B cells and it synergizes with IL-1, IL-3, and GM-CSF to enhance the proliferation and growth of hematopoietic progenitors in human beings.²⁷⁻²⁹ It has been recently shown that IL-6 promotes the survival of human mast cells in vitro and enhances IgE-mediated histamine release.³⁰ IL-6 is synthesized and stored in unstimulated eosinophils from normal donors and donors with allergies. In a recent study we have demonstrated by subcellular fractionation, confocal laser scanning microscopy, and immunocytochemical staining that IL-6 is stored in the secretory granules of eosinophils.¹⁶ In addition, IL-6 is contained in discrete quantities (25 pg/10⁶ cells) and can also be released by incubation of eosinophils with IFN- γ .¹⁶

Interestingly, IL-6 is contained in the same eosinophil secretory granules as GM-CSF, IL-2, and IL-5.^{15,16} Therefore the lack of IL-6 release from eosinophils after incubation with NGF may suggest that NGF also has no effect on the secretion of other cytokines and especially eosinophil survival, cytokines in GM-CSF, IL-3, and IL-5,³¹ and hence no role in eosinophil survival. Indeed, in the next series of experiments, we could not find any enhancement of NGF on eosinophil viability.

Our data of absence of NGF survival activity on human eosinophils stand in contrast with the results of a recent study, in which eosinophils cultured with NGF (50 ng/mL) resulted in 50% enhancement of their viability.¹⁸ This discrepancy in results might possibly be explained by the degree of purification (which was 70% to 80% in that study compared with more than 98% in our study) and the different experimental conditions. In addition, despite the reports of the effects of NGF on the survival of rodent mast cells,³² on mature sympathetic neurons,³³ on memory B lymphocytes,³⁴ and on neutrophils,³⁵ human mast cells reportedly do not respond to NGF.^{8,9} In fact, they do not respond to NGF even though it has been recently reported that they express Trk-A receptors and are a source of NGF.⁸

NGF has been found to be produced by rodent and human mast cells⁸⁻¹⁰ and more recently by memory B lymphocytes.³³ We therefore wondered whether eosinophils could also contain preformed NGF as in these cells. To this end, we evaluated NGF content in sonicates of freshly isolated eosinophils. It was found that eosinophils contain variable levels of NGF. In addition, to confirm that eosinophils produce NGF, reverse transcription PCR was performed on RNA isolated from freshly isolated eosinophils. These experiments showed the presence of mRNA for NGF in eosinophils and therefore their ability to produce and store NGF. An autocrine mechanism for NGF has been suggested for mast cells and B lymphocytes.⁸⁻¹⁰ The production of NGF by eosinophils and its effects on these cells implicates a possible autocrine mechanism for eosinophils as well.

In conclusion, both the data from the literature showing that NGF levels are increased in allergic inflammation and that mast cells and CD4⁺ cells produce, store, and respond to NGF, together with our present data showing that NGF activates eosinophils and is stored and produced by these cells, suggest an important role for NGF in allergic inflammation. Because NGF causes EPO release and probably release of other basic proteins that are known to be toxic to tissues, anti-NGF therapy in allergy may be possible in the future. It is therefore important to perform further studies to define NGF activity in allergy as a potential target for the immunopharmacologic modulation in allergic diseases.

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REFERENCES

1. Callissano P, Cattaneo A, Aloe L, Levi-Montalcini R. The nerve growth factor (NGF). In: Hormonal proteins and peptides. Vol XII. New York: Academic Press; 1984. p 1-56.

2. Thoenen H, Edgar D. Neurotrophic factors. *Science* 1995;229:238.
3. Aloe L, Bracci-Laudiero L, Bonini S, Manni L. The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases. *Allergy* 1997;52:883-994.
4. Aloe L, Levi-Montalcini R. Mast cells increase in tissues of neonatal rats injected with nerve growth factor. *Brain Res* 1977;133:358-66.
5. Marshal JS, Stead RH, McSharry C, Nielsen L, Bienenstock J. The role of mast cell degranulation products in mast cell hyperplasia, I: mechanism of action of nerve growth factor. *J Immunol* 1990;144:1886-92.
6. Matsuda H, Kannan Y, Ushio H, Kiso Y, Kanemoto T, Suzuki H, et al. Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. *J Exp Med* 1991;174:7-14.
7. Horigome K, Pryor JC, Bullock ED, Johnson EM Jr. Mediator release from mast cells by nerve growth factor: neurotrophin specificity and receptor mediation. *J Biol Chem* 1993;268:14881-7.
8. Nilsson G, Forsberg-Nilsson K, Xiang L, Hallbook F, Nilsson K, Metcalfe DD. Human mast cells express functional Trk A and are a source of NGF. *Eur J Immunol* 1997;27:2295-301.
9. Tam S-Y, Tsai M, Yamaguchi M, Yano K, Butterfield JH, Galli SJ. Expression of functional Trk A receptor Tyrosine Kinase in the HMC-1 human mast cell line and in human mast cells. *Blood* 1997;90:1807-20.
10. Leon A, Buriani A, Dal Toso R, Fabris M, Romanello S, Aloe L, et al. Mast cells synthesize, store, and release nerve growth factor. *Proc Natl Acad Sci U S A* 1994;91:3734-9.
11. Bonini S, Lambiase A, Bonini S, Angelucci L, Magrini L, Aloe L. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc Natl Acad Sci U S A* 1996;93:10955-60.
12. Lambiase A, Bonini ST, Bonini SE, Micera A, Magrini L, Bracci-Laudiero L, et al. Increased plasma levels of nerve growth factor in vernal keratoconjunctivitis and relationship to conjunctival mast cells. *Invest Ophthalmol Vis Sci* 1995;36:2127-32.
13. Lambiase A, Bracci-Laudiero L, Bonini SE, Bonini ST, Starace G, D'Elios MM, et al. Human CD4⁺ T-cell clones produce and release nerve growth factor (NGF) and express high affinity NGF receptor. *J Allergy Clin Immunol* 1997;100:408-14.
14. Moqbel R, Levi-Schaffer F, Kay AB. Cytokine generation by eosinophils. *J Allergy Clin Immunol* 1994;94:1103-89.
15. Levi-Schaffer F, Lacy P, Severs NJ, Newman TM, North J, Gomperts B, et al. Association of granulocyte-macrophage colony-stimulating factor with the crystalloid granules of human eosinophils. *Blood* 1995;85:2576-9.
16. Lacy P, Levi-Schaffer F, Mahmudi-Azer S, Babeitz B, Hajen SC, Velazquez J, et al. Intracellular localization of interleukin-6 in eosinophils from atopic asthmatic and effects of interferon- γ . *Blood* 1998;91:2508-16.
17. Takafuji S, Bischoff SC, De Weck AL, Dahinden CA. Opposing effects of tumor necrosis factor- α and nerve growth factor upon leukotriene C4 production by human eosinophils triggered with N-formyl-methionyl-leucyl-phenylalanine. *Eur J Immunol* 1992;22:969-74.
18. Hamada A, Watanabe N, Ohtomo H, Matsuda H. Nerve growth factor enhances survival and cytotoxicity of human eosinophils. *Br J Hematol* 1996;93:299-302.
19. Lambiase A, Bonini ST, Micera A, Rama P, Bonini SE, Aloe L. Expression of NGF receptors on ocular surface in healthy subjects, and during inflammatory diseases. *Invest Ophthalmol Vis Sci* 1998;39:1272-5.
20. Bocchini V, Angeletti PU. The nerve growth factor: purification as a 30,000 molecular weight protein. *Proc Natl Acad Sci U S A* 1969;64:787-94.
21. Levi-Montalcini R. The nerve growth factor 35 years later. *Science* 1987;237:1154-62.
22. White SR, Kulp GVP, Spaethe SM, Van Alstyne E, Leff AR. A kinetic assay for eosinophil peroxidase activity in eosinophils and eosinophil conditioned media. *J Immunol Methods* 1991;144:257-63.
23. Bracci-Laudiero L, Aloe L, Levi-Montalcini R. Multiple sclerosis patients express increased levels of β -nerve growth factor in cerebrospinal fluid. *Neurosci Lett* 1992;147:9-12.
24. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate phenol-chloroform extraction. *Anal Biochem* 1987;162:156.
25. Cartwright M, Mikheev AM, Heinrich G. Expression of neurotrophic genes in human fibroblasts: differential regulation of the brain-derived neurotrophic factor gene. *Int J Dev Neurosci* 1994;12:685-93.
26. Tomassini M, Tsicopoulos A, Tai PC, Gruart V, Tonnel AB, Prin L, et al. Release of granule proteins by eosinophils from allergic and nonallergic

- patients with eosinophils on immunoglobulin-dependent activation. *J Allergy Clin Immunol* 1991;88:365-75.
27. Vercelli D, Jabara HH, Arai K, Yokota T, Geha RS. Endogenous IL-6 plays an obligatory role in IL-4 induced human IgE synthesis. *Eur J Immunol* 1989;18:929.
 28. Leary AG, Ikebuchi K, Hirai Y, Wong GG, Yang YC, Clark SC, et al. Synergism between interleukin-6 and interleukin-3 in supporting proliferation of human hematopoietic stem cells: comparison with interleukin-1 α . *Blood* 1988;71:1759.
 29. Caracciolo D, Clark SC, Rovera G. Human interleukin-6 supports granulocytic differentiation of hematopoietic progenitor cells and acts synergistically with GM-CSF. *Blood* 1989;73:666.
 30. Yanagida M, Fukamachi H, Ohgami K, Kuwaki T, Ishii H, Uzumaki H, et al. Effects of T-helper 2-type cytokines interleukin 3 (IL-3), IL-4, IL-5 and IL-6 on the survival of cultured human mast cells. *Blood* 1995;86:3705-14.
 31. Tai PC, Sun L, Spry CJ. Effects of IL-5, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-3 on the survival of human blood eosinophils in vitro. *Clin Exp Immunol* 1991;85:312-6.
 32. Kawamoto K, Okada T, Kannan Y, Ushio H, Matsumoto M, Matsuda H. Nerve growth factor prevents apoptosis of rat peritoneal mast cells through the Trk proto-oncogene receptor. *Blood* 1995;86:4638-44.
 33. Greenlund LJS, Korsmeyer SJ, Johnson EM. Role of BCL-2 in the survival and function of developing and mature sympathetic neurons. *Neuron* 1995;15:601-49.
 34. Torcia M, Bracci-Laudiero L, Lucibello M, Nencioni L, Labardi D, Rubartelli A, et al. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell* 1996;85:345-56.
 35. Kannan Y, Usami K, Okada M, Shimizu S, Matsuda H. Nerve growth factor suppresses apoptosis of murine neutrophils. *Biochem Biophys Res Commun* 1992;186:4638-44.

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