Effect of Nerve Growth Factor on the Release of Inflammatory Mediators by Mature Human Basophils

By Stephan C. Bischoff and Clemens Dahinden

Nerve growth factor (NGF) is a neurotrophic cytokine known to regulate the survival and function of peripheral and central neuronal cells. Recently, the spectrum of action could be extended to non-neuronal cell types such as rat mast cells and human B lymphocytes. The present study shows that NGF affects the function of mature human basophils isolated from the peripheral blood of healthy donors. Both murine NGF 7S and recombinant human NGF β enhance histamine release and strongly modulate the formation of lipid mediators by basophils in response to various stimuli. This priming effect of NGF on basophils occurs rapidly within 10 to 15 minutes of preincubation, is dose-dependent, and requires similarly low concentrations (1 to 40 pmol/L) of human NGF β as the induction of neutre outgrowth in ganglion cells. Cell fractionation studies indicate that NGF acts directly on human basophils without an involvement of other cell types, suggesting the presence of high-affinity NGF receptors on basophils. NGF by itself (up to 4 nmol/L of human NGF β) does not induce the release of inflammatory mediators directly. The effect of human NGF on basophil mediator release is similar to that of the hematopoietic growth factors interleukin-3, interleukin-5, and granulocyte-macrophage colony-stimulating factor. The present study further demonstrates that NGF acts as a pleiotropic cytokine at the interface between the nervous and the immune system, and that NGF may be involved in inflammatory processes and hypersensitivity reactions.

© 1992 by The American Society of Hematology.

From the Institute of Clinical Immunology, Inselspital, Bern, Switzerland.


Supported by the Swiss National Science Foundation (Grant No. 31 27980.89). S.C.B. is supported by a grant from Deutsche Forschungsgemeinschaft, Germany.

Address reprint requests to Stephan C. Bischoff, MD, Institute of Clinical Immunology, Inselspital, University Hospital of Bern, CH-3010 Bern, Switzerland.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

Reagents. EDTA and sodium azide were purchased from Fluka AG (Buchs, Switzerland); Dextran T70, Ficoll Hypaque, and Percoll were from Pharmacia Fine Chemicals (Uppsala, Sweden); HEPES was from Calbiochem-Behring Corp (La Jolla, CA); bovine serum albumin (BSA), fatty acid free, was from Boehringer Mannheim, Inc (Mannheim, Germany); gelatin and activated charcoal were from Sigma Chemical Company (St Louis, MO); and Dynabead M-450 coated with anti-CD2 (Pan T), anti-CD19 (Pan B), or sheep antimouse IgG1 antibodies (Ab) were from Dynal A.S. (Skøyen, Norway). Sheep antimouse Ab-coated beads (4 × 10⁵) were incubated for 24 hours with 25 μg mouse monoclonal anti-CD16 Ab (kindly provided by Dr V. Kurrle, Behring, Marburg, Germany) to obtain specific beads directed against neutrophils and natural killer (NK) cells.

Cell preparation. Basophils were prepared from venous blood of healthy human volunteers as described in detail previously.³⁴
For most experiments, leukocytes were separated from erythrocytes by dextran-sedimentation and fractionated by Ficoll Hypaque density centrifugation (400g for 40 minutes at room temperature). The basophil-containing mononuclear cell layer was washed, harvested, and finally suspended at a density of 2.5 to 5 × 10⁶ cells/mL in HACM-buffer (20 mmol/L HEPES, 125 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L glucose, 0.025% BSA, 1 mmol/L CaCl₂, and 1 mmol/L MgCl₂). These preparations contained 1% to 6% (mean, ±2%) basophils as determined by stained cytocentrifuge slides. In some experiments, leukocytes were fractionated on discontinuous Percoll gradients (densities: 1.079, 1.070, and 1.062 containing the leukocytes) and centrifuged (500g for 30 minutes at room temperature). The cell layers at the interface between the 1.070 and 1.062 gradient (4% to 10% basophils) and between the 1.079 and 1.070 gradient (20% to 50% basophils) were harvested and the latter was depleted of contaminating T cells, B cells, NK cells, and neutrophils using immunomagnetic beads (cell/beads ratio = 1:10) coated with mouse anti-CD2 (60%), anti-CD19 (10%), and anti-CD16 monoclonal Ab (MoAb) (30%). The resulting basophil preparation (purity, 85% to 96%) was washed twice in HA-buffer and finally resuspended in HACM-buffer at a density of 1 to 2 × 10⁶ cells/mL.

**Cell stimuli.** The β-form of rhuNGF, expressed in Chinese hamster ovary cells and purified to greater than 98%, was a generous gift from Genentech, Inc (South San Francisco, CA). The biologic activity of rhuNGF (molecular weight [MW] of the dimer, 25.6 Kd) was determined by neurite formation in PC12 cells (a rat pheochromocytoma cell line). A half-maximal activity in this assay was produced by 1.5 to 2 pmol/L rhuNGF.23 Marine NGF 75 complex (muNGF 75, MW = 140 Kd), prepared from male mouse salivary glands and purified to 99% as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was from Boehringer Mannheim, Inc. MuNGF 75 activity was determined by fiber outgrowth of chick embryo dorsal root ganglia and became maximal at 400 to 700 pmol/L. The lyophilized preparation was reconstituted according to the manufacturers instructions and further diluted in HA buffer containing 1 mg/mL BSA. Dr Y.A. Barde (Max-Planck-Institute of Psychiatry, Planegg-Martinsried, Germany) kindly provided us with COS-conditioned medium containing recombinant brain-derived neurotrophic factor (BDNF). Final dilutions of 1:100 to 1:300 were maximally active in promoting the survival of peripheral nervous sensory neurons isolated from chick embryos.24 rhuIL-3 and rhuGM-CSF were from Sandoz (Basel, Switzerland). Supernatants from Escherichia coli cultures expressing the human IL-5 protein were a generous gift from Dr C.J. Sanderson (National Institute for Medical Research, London, UK). These supernatants contained basophil mediator release (maximal effect at a dilution of 1:1,000) as the purified preparation.4 The complement cleavage product C5a was purified from yeast-activated human serum as described.3 FMLP was from Fluka AG (Buchs, Switzerland); C18 platelet-activating factor (PAF) was from Novabiochem (Läuelfingen, Switzerland). Anti-IdGE LE27 MoAb was prepared as described.4

**Mediator release assay.** The experiments were performed in a thermostat-regulated (37°C) shaking water bath. In most experiments, the cells were preincubated without or with cytokines (NGF, BDNF, IL-3, IL-5, GM-CSF) during 10 minutes and then stimulated with a triggering agent (CSa, anti-IdGE Ab, FMLP) for 20 minutes, a time sufficient for maximal mediator release, as determined in earlier studies.24 The reaction was stopped by cooling the cell suspension on ice. Supernatants were obtained by centrifugation (400g for 10 minutes at 4°C) and divided into two parts: 0.4 mL were directly stored at −70°C for leukotriene measurement, 0.5 mL were mixed with 0.5 mL of HACM buffer and 1 mL of HClO₄, centrifuged (600g for 15 minutes at 4°C), and these supernatants were also stored at −70°C for histamine measurement using an automated fluorometric method.3

**Leukotriene measurement.** The sLT including LTC4/D4/E4 were measured by a fluid phase radioimmunoassay (RIA) based on competition between unlabeled LTC4 (standards or samples) and a fixed quantity of tritium-labeled LTC4 (tracer) for binding to a limited quantity of MoAb directed against sLT. The LTC4 tritium tracers, either [14,15,19-3H]LTC4 (specific activity, 39.3 Ci/mmol) or the higher labeled [14,15,19,20-3H]LTC4 (168.4 Ci/mmol), were from NEN research products (Boston, MA). Tracers were diluted in assay buffer (phosphate-buffered saline [PBS] 0.05 mol/L, pH 7.4, containing 10 mg/mL gelatine) to 1.77 and 0.38 pmol/mL, respectively. LTC4 standards (Amersham Corp, Arlington, IL) were diluted to 0.03 to 8 ng/mL in HACM buffer. The mouse MoAb against sLT (sLT-Ab) was kindly provided by Drs J. Möllenhauer and K. Brune (University of Erlangen-Nürnberg, Germany). This Ab had the same sensitivity for LTC4, LTD4, and LTE4, and showed no cross-reactivity to LTβ4 and its metabolites, to prostaglandins D₂, E₂, F₂α, or to thromboxane B₂.25 The hylophilized unpurified sLT-Ab from cell culture supernatant was reconstituted in PBS/H₂O 1:1 (1 mg of total protein/mL), and further diluted in assay buffer (1:100 for the less sensitive assay, 1:300 for the high sensitive assay, determined to be optimal for each assay). One hundred microliters of supernatants or of standard LTC4 solutions + 100 μL of LTC4 tracer + 100 μL of sLT-MoAb solution + 100 μL of HACM buffer (400 μL in total per tube) were mixed. After overnight incubation at 4°C, 250 μL of charcoal suspension (assay buffer + 300 mg/mL charcoal, 160 mg/mL dextran T70, 1 mg/mL sodium azide) was added for 15 minutes to absorb unbound LTC4 tracer. Tubes were then centrifuged (2,000g for 15 minutes at 4°C), and 450 μL of the supernatants was transferred into scintillation vials. The amount of LTC4 tracer bound to the Ab was quantified in the β-counter and was inversely proportional to the concentration of unlabeled sLT. ‘Zero standard’ (without unlabeled LTC4) was 1,200 to 1,500 cpm (corresponding to 25% to 35% binding of the tracer to the antibody). ‘Nonspecific binding’ (zero standard without antibody addition) was less than 100 cpm. sLT from 12.5 to 800 pg/tube and from 3.125 to 400 pg/tube, respectively, dependent on the assay used for measurement, could be detected (Fig 1). Measurements were identical to those performed with a commercially available LTC4/D4/E4 RIA kit (Amersham Corp) following the manufacturers protocol, despite the fact that the polyclonal antiserum used in this assay recognizes LTD4 and LTE4 by only 64% as compared with LTC4. This is due to the negligible conversion of LTC4 to LTD4/E4 in mononuclear cells under incubation conditions used in this study, as we have shown previously by high performance liquid chromatography (HPLC).2 Nevertheless, the equal affinity of the sLT-Ab for all sLT is preferable because total sLT are measured, and results are not influenced by possible variations in LTC4 metabolism.

**Presentation of data.** All experiments were performed at least in duplicate, and repeated three times or more. Histamine data are presented as mean of percent specific histamine release according to the formula 100 × [release - spontaneous release]/[total histamine content]. Leukotriene data are expressed as mean of picograms of leukotriene per nanogram of total histamine content per tube to correct for different basophil numbers.2 Data derived from several experiments are usually presented as mean ± standard error of the mean (SEM). Mediator release values in response to the same triggering agent within the same cell preparation but with different preincubation conditions (Figs 2 and 3) were analyzed by analysis of variance and the paired t-test according to Sachs.26

---

RESULTS

Figure 2 shows that preincubation of basophils for 10 minutes with either rhuNGF β or muNGF 7S enhanced concentration-dependent histamine release as well as the generation of leukotrienes in response to anti-IgE Ab or FMLP. Apart from these enhancing effects, both NGF preparations also qualitatively changed the release profile in response to C5a, which by itself induced degranulation only. NGF rendered basophils capable of synthesizing lipid mediators in response to C5a, while histamine release was similarly enhanced as the response to FMLP or anti-IgE Ab. Figure 2 shows that this priming effect of rhuNGF β and muNGF 7S on human basophils occurred within a concentration range of 0.03 to 1 ng/mL and 10 to 100 ng/mL, reaching a plateau at 1 to 10 ng/mL and 100 to 1,000 ng/mL, respectively. Thus, basophils are influenced by rhuNGF β (ED50 = 4 pmol/L) and muNGF 7S (ED50 = 140 pmol/L) at concentrations similar to those required for stimulation of neuronal cells. Importantly, neither rhuNGF β nor muNGF 7S did induce any mediator release in basophils by themselves, up to concentrations of 100 ng/mL rhuNGF β and 1,000 ng/mL muNGF 7S, respectively (data not shown). By contrast, BDNF, a neurotrophic protein structurally related to NGF, failed to induce or modulate basophil mediator release at concentrations determined to be optimal for promoting survival of chick embryo sensory neurons (1:1,000 to 1:100 dilutions).

There is a remarkable, well-known donor variability of basophil mediator release in response to anti-IgE Ab or FMLP, and to a lesser extent to C5a. Thus, in a large number of experiments, cells from different donors were preincubated in buffer control or with optimal concentra-

tions of muNGF 7S, rhuNGF β, or rhuIL-3 within the same experiment. After 10 minutes, cells were triggered with maximally effective concentrations of C5a, FMLP, or anti-IgE Ab (Fig 3). IL-3 was included in these experiments, because we found in previous studies that, within the HGF capable of modifying basophil function, IL-3 is the most active cytokine. Figure 3 shows the variability of histamine and leukotriene release, respectively, from experiments performed with different donors, as well as mean values from the different stimulation protocols. Both muNGF 7S and rhuNGF enhanced histamine release and sLT generation in response to C5a, anti-IgE Ab, and FMLP. Furthermore, in all experiments, NGF-primed basophils produced large amounts of sLT in response to C5a, while, with the exception of rare cases, no sLT were detected from cells stimulated with C5a alone. It should be noted that in all experiments mediator release in response to the three agonists was enhanced by NGF preincubation, when compared with the corresponding control value (paired t-test, all P < .001). The enhancement of mediator release due to
optimal concentrations of murine or human NGF did not differ significantly (all \( P > .05 \)). However, mediator release, in particular in response to anti-IgE stimulation, was less pronounced after NGF preincubation as compared to IL-3. The reason for the different effects of NGF and IL-3 is not yet understood, but may be due to the necessity of dissociation of the NGF 7S complex to become active for basophil priming.

In three experiments, highly purified basophil preparations (85% to 96%) were used to determine the participation of other leukocyte types in the action of NGF on basophils. We also performed experiments with leukocyte preparations fractionated by Percoll gradients containing basophils of different densities and variable proportions of other leukocyte types. Figure 5A shows a representative experiment performed with a cell preparation of 96% basophil purity (contaminating cells were small lymphocytes). The data show that C5a- and anti-IgE Ab-induced mediator release was enhanced by rhuNGF. NGF-primed, but not unprimed, purified basophils release large amounts of sLT in response to C5a. The data were not different when, within the same experiment of the same cell fraction, depletion of T cells, B cells, NK cells, and neutrophils was

<table>
<thead>
<tr>
<th>C5a</th>
<th>Anti-IgE Ab</th>
<th>FMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hist.</td>
<td>LTC4</td>
<td>Hist.</td>
</tr>
<tr>
<td>Control</td>
<td>39 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>rhuNGF</td>
<td>59 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>rhuIL-3</td>
<td>64 ± 2</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>rhuIL-5</td>
<td>65 ± 2</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>rhuGM-CSF</td>
<td>63 ± 2</td>
<td>31 ± 4</td>
</tr>
</tbody>
</table>

Basophils were preincubated within the same experiment with either rhuNGF (1 ng/mL), rhuIL-3 (10 ng/mL), or rhuIL-5 (1:1000 dilution), or rhuGM-CSF (10 ng/mL), or without cytokines for 10 minutes before addition of C5a (10^(-4) mol/L), anti-IgE Ab (100 ng/mL), or FMLP (2.5 x 10^(-6) mol/L). Data are expressed as mean ± SEM (n = 3) of percent histamine release (Hist.) or picograms of sLT per nanogram of total histamine (LTC4) from three experiments performed in duplicate.
omitted (Fig 5B). The pattern of mediator release and the influence of NGF was identical in low density basophils derived from an upper cell layer (between 1.070 and 1.062 density gradients), mainly contaminated with lymphocytes. These cell preparations produced slightly larger amounts of leukotrienes, and in the supernatants of cells triggered by C5a alone some sLT was detected (Fig 5C). We cannot decide at present whether functional differences exist within basophils of different densities, or whether the discretely larger amounts of sLT produced in the cell fractions of lower density are due to contaminating leukocytes, because we have not yet achieved a complete purification of hypodense basophils. However, the data clearly show that the modulating effect of NGF is independent of a contamination with other leukocyte types.

**DISCUSSION**

The present study shows that human NGF at very low concentrations influences the function of one type of mature human effector cells, and shows a first biologic activity of pure rhuNGF p-chain outside the nervous system. This priming effect of NGF, which occurs when basophils have been preincubated for a few minutes in the presence of the neurotrophic cytokine, consists of an enhancement of histamine and sLT release induced by FMLP or anti-IgE Ab, or of a qualitative modification of the basophil response towards C5a, in which case NGF preincubation is required for lipid mediator synthesis.

Previous work with rat peritoneal mast cells showed that purified muNGF directly induces histamine release in a dose-dependent manner. However, degranulation occurred only at high concentrations (5 to 500 nmol/L of NGF), or in the presence of lysophospholipids.13-16 Furthermore, muNGF 2.5S induced a shape change in rabbit platelets at a concentration of 570 nmol/L.17 Such concentrations are...
NGF AFFECTS HUMAN BASOPHILS

orders of magnitude above that required in ganglion or PC12 cell assays. In vivo, 4 to 400 pmol of muNGF 2.5S induced plasma extravasation in rat skin, which could be reduced by capsaicin pretreatment and by histamine antagonists, indicating an involvement of both a neurogenic (eg, via substance P) and a basophil/mast cell-dependent mechanism.27 By contrast, in the human system NGF is not a direct basophil agonist, because it induces no mediator release by itself, even at concentrations up to 100 ng/mL of rhuNGF, or up to 1 µg/mL of muNGF 7S. Furthermore, in the presence of IL-3, in which case several otherwise inactive basophil triggers (C3a, IL-8, platelet-activating factor) efficiently induce histamine and sLT release,28,29 NGF is still ineffective (our unpublished results).

The effect of NGF on mature human basophils is remarkably similar to that of IL-3. In the mouse, IL-3 acts as a mast cell growth factor, while in human bone marrow cultures IL-3 induces proliferation and differentiation of myeloid progenitors into immature histamine-containing cells, which resemble basophils more than mast cells.30 Furthermore, in contrast to basophils, human mucosal mast cells do not bear IL-3 receptors, and their function is not affected by IL-3.31,32 Interestingly, one of the earliest and most firmly established effects of NGF outside the nervous system in rats is its capacity to cause an increase in size and number of mast cells in several peripheral tissues,30 possibly through an autocrine involvement of mast cell product release.34 In the human system, however, NGF neither modulates nor induces granule release in human mucosal mast cells.32 Therefore, the biologic effects of NGF and IL-3 seem to be quite similar in the human as well as in the mouse system, except that the target cells, basophils, and mast cells, are different.

Among a large number of cytokines examined, only IL-3, IL-5, and GM-CSF were found to modulate the function of mature human basophils.2,4 Thus, NGF is the first basophil response modifier, which was not primarily described as a HGF. Of interest, Matsuda et al showed that muNGF also acts on human myeloid progenitor cells as a growth and differentiation factor in particular of the eosinophil and basophil/mast cell lineages.20,21 However, in contrast to the HGF properties of IL-3, GM-CSF, and IL-5, which affect the myeloid cells directly, this biologic activity of NGF appeared to be T-cell-dependent. Because of the similarity of biologic effects of these HGFs and NGF on basophils as well as on myeloid progenitors, it is tempting to speculate that NGF stimulates the progenitor cells directly, but in synergy with a T cell product(s). Indeed, recent data showed that NGF promotes human granulopoiesis only in synergy with GM-CSF.22

The NGF effect on basophils is rapid and is independent of the degree of contamination by other leukocyte types, suggesting a direct effect of NGF on basophils through specific receptors. Sutter et al15 described two classes of NGF receptors (NGFR), a high-affinity receptor (kd = 23 pmol/L) probably mediating nerve cell survival and neurite outgrowth, and a low-affinity receptor (kd = 1.7 nmol/L), which seems to be required but not sufficient for functional expression of high-affinity receptors.36 The low-affinity NGFR has been cloned in human, rat, and chicken, and binds NGF and even the related BDNF with equal affinity.12,37 Very recent studies indicate that the trk proto-oncogene either by itself or in combination with the low-affinity NGFR forms the high-affinity NGFR in neuronal cells.38,39 There is strong evidence that the biologic effect of NGF at picomolar concentrations on primary neurons of animal origin as well as on human basophils, as shown here, is mediated by a high-affinity NGFR.7,39 It is less clear whether the higher NGF concentrations required to affect human B cells and myeloid progenitor cells (ED50 = 10 to 50 mg/mL = 0.4 to 2 nmol/L)18-22 are due to the lack of high-affinity binding sites on these target cells or to a weaker bioactivity of muNGF on human cells. The low-affinity NGFR belongs to a family of structurally related membrane proteins including CD40, which has been regarded as a specific leukocyte marker of B cells.40,41 Recent studies, however, have shown that human basophils, but not mast cells, also express CD40.42 Interestingly, in humans, B lymphocytes and basophils both respond to NGF and express CD40, but whether this association is related to the biologic effects of NGF on these two cell types is unknown.

NGF belongs to an increasingly large family of neurotrophic proteins, which are strongly homologous in primary structure.34,43 Three family members have been identified, NGF, BDNF and neurotrophin 3 (NT3), with partially overlapping, partially distinct biologic activities on neuronal cells. It has been suggested that they could interact with a common binding protein, the low-affinity NGFR,43 possibly in association with accessory proteins creating high-affinity states specific for each neurotrophic factor.36,39 Our own preliminary data indicate that BDNF does not affect basophil function at concentrations promoting survival of nodose neurons, further supporting the hypothesis of a high-affinity NGFR on basophils. Clearly, additional studies are needed to define the functional effects of other neurotrophic factors on basophils.

muNGF 7S and rhuNGF β prime basophils at a concentration range of 70 to 700 pmol/L and 1 to 40 pmol/L, respectively. These concentrations of both NGF preparations correspond well to those required to affect neuronal cells.2,23 The extremely high biologic activity of rhuNGF β on basophils indicates that the bioactivity of muNGF 7S is due to the β-subunit. A definitive assessment of possible differences of bioactivity between mouse and human NGF β on human basophils would require a direct comparison of recombinant purified murine and human material under identical experimental conditions.

Our results extend and confirm the concept that NGF affects both the nervous and the immune system. However, the pathophysiologic significance of this interface between nervous and immune system, the site of NGF production, and the regulation of NGF during health and disease is still unclear.7,19,18 In particular, informations on human tissue containing NGF are rare and controversial, in part due to difficulties and misinterpretations of NGF measurements by immunoassays or biologic assays.7 Nevertheless, it is tempting to speculate that NGF is synthesized locally in
many tissues, possibly under the control of other cytokines, and may be involved in different pathologic conditions such as tumor growth, cell regeneration, tissue destruction, and wound healing, or in neurogenic or allergic inflammatory processes.

ACKNOWLEDGMENT

We thank Dr G. Burton and colleagues (Genentech, Inc, San Francisco, CA), Dr C.J. Sanderson (London, UK), Dr Y.A. Barde (Planegg-Martinsried, Germany), Dr M. Schreier (Sandoz, Basel, Switzerland), and Dr E. Liehl (Vienna, Austria) for providing us with recombinant human cytokines, and Dr V. Kurrle (Behring, Marburg, Germany), Dr K. Brune, and Dr J. Mollenhauer (Erlangen, Germany) for monoclonal antibodies. The authors gratefully acknowledge the help of Dr B. Neuenschwander (Institute of Mathematics, University of Bern, Switzerland) in statistical analysis, and the technical assistance of A. Raafraub-Sohn, J. Zingg, and P. Winkler.

REFERENCES

41. Stamenkovic I, Clark EA, Seed B: A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J 8:1403, 1989
Effect of nerve growth factor on the release of inflammatory mediators by mature human basophils

SC Bischoff and CA Dahinden