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Nerve growth factor promotes human hemopoietic colony growth and differentiation

(myelopoiesis/basophils/mast cells/T cells/allergy/eosinophils)

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ABSTRACT **Nerve growth factor (NGF) is a neurotropic polypeptide necessary for the survival and growth of some central neurons, as well as sensory afferent and sympathetic neurons. Much is now known of the structural and functional characteristics of NGF, whose gene has recently been cloned. Since it is synthesized in largest amounts by the male mouse submandibular gland, its role exclusively in nerve growth is questionable. NGF also causes histamine release from rat peritoneal mast cells *in vitro*, and we have shown elsewhere that it causes significant, dose-dependent, generalized mast cell proliferation in the rat *in vivo* when administered neonatally. Our experiments now indicate that NGF causes a significant stimulation of granulocyte colonies grown from human peripheral blood in standard hemopoietic methylcellulose assays. Further, NGF appears to act in a relatively selective fashion to induce the differentiation of eosinophils and basophils/mast cells. Depletion experiments show that the NGF effect may be T-cell dependent and that NGF augments the colony-stimulating effect of supernatants from the leukemic T-cell (Mo) line. The hemopoietic activity of NGF is blocked by polyclonal and monoclonal antibodies to NGF. We conclude that NGF may indirectly act as a local growth factor in tissues other than those of the nervous system by causing T cells to synthesize or secrete molecules with colony-stimulating activity. In view of the synthesis of NGF in tissue injury, the involvement of basophils/mast cells and eosinophils in allergic and other inflammatory processes, and the association of mast cells with fibrosis and tissue repair, we postulate that NGF plays an important biological role in a variety of repair processes.**

Nerve growth factor (NGF) is a well-characterized neurotrophic polypeptide synthesized in parts of the brain and nervous system. The amino acid sequence is established, with greater than 90% identity existing between the murine and human forms (1–4). NGF is necessary for the development and function of sympathetic and embryonic sensory cells, and its biological activity is mediated through interaction with specific receptors found on normal or neoplastic target cells derived from the neural crest. Despite its name, NGF may have broader biological functions, placing it in the family of peptide growth factors. For example, NGF causes degranulation of rat peritoneal mast cells (5), induces shape changes in platelets (6), and accelerates wound healing (2, 7). Since NGF injections *in vivo* cause mast cell numbers to increase in various tissues of neonatal rats (8–11), we explored the effect of NGF on human hemopoietic colony formation, with particular emphasis on basophil/mast cell colonies.

We have previously shown that basophil/mast cell progenitors, a subpopulation of eosinophil colony-forming cells (12), circulate in normal human subjects (13), are increased in atopic subjects (14), and are found in the nasal mucosa of patients with nasal polyps (15–17). Basophil/mast cell and eosinophil differentiation-promoting factors are produced by human nasal polyp and allergic rhinitis mucosal epithelial scrapings *in vitro*, suggesting the importance of locally derived factors in the regulation of numbers of these specialized granulocytes in allergic inflammation (17, 18). We have also shown extensive mast cell/nerve structural membrane–membrane associations in normal and inflamed rat intestines (9, 10). Mast cell/nerve interactions may be important in various forms of neurogenic inflammation, *in vivo* and in the homeostatic regulation of various internal physiologic parameters (19–22). We now hypothesize that NGF plays an accessory role in the regulation of basophil/mast cell growth and differentiation in allergic and nonallergic inflammatory processes.

MATERIALS AND METHODS

Hemopoietic Cell Assays and Cultures. Mononuclear cells were separated from heparinized, normal human peripheral blood and plated in methylcellulose cultures. After macrophages/monocytes were removed by adherence to plastic at 37°C for 2 hr, nonadherent mononuclear cells (NAMC) were cultured in methylcellulose. Briefly, 1×10^6 NAMC were cultured in 0.9% methylcellulose in Iscove's modified Dulbecco's medium (GIBCO), 20% fetal calf serum (GIBCO), penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 5 μM 2-mercaptoethanol, with or without 5% (vol/vol) conditioned medium (CM) derived from a human T-cell line (Mo-CM) at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. On the 14th day of culture, granulocyte and macrophage colonies made up of more than 40 cells were counted and classified into neutrophil–macrophage (GM), eosinophil (Eo), or macrophage (M) type. Individual colonies (10 Eo-type and 6 GM-type) were randomly picked from methylcellulose medium by a Pasteur pipette with the aid of an inverted microscope, placed onto glass slides, and stained with May–Grunwald Giemsa for morphological evaluation and differential counting. A colony with more than 5% of polymorphonuclear leukocytes containing basophilic granules in their cytoplasm was classified as a basophil/mast cell colony and almost invariably represented a subtype of Eo-type colonies (12–16). Colony-stimulating activity (CSA) was defined as the mean number of granulocyte colonies of a given type per 10^6 cells plated in quadruplicate cultures at day 14, and it was subcategorized as GM-CSA, Eo-CSA, or

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Abbreviations: NGF, nerve growth factor; CM, conditioned medium; GM, neutrophil–macrophage; Eo, eosinophil; M, macrophage; NAMC, nonadherent mononuclear cells; CSA, colony-stimulating activity.

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M-CSA, depending on the type of colonies stimulated in the presence of a given concentration of CM.

Preparation and Isolation of NGF. NGF was isolated in the 2.5S form from murine submaxillary glands according to methods previously described (23–27). NGF was further purified by gel filtration on a Sephadex G-75 column (0.7 × 90 cm) in 2 M acetic acid. This additional gel filtration step serves to remove traces of renin and IgG sometimes found as contaminants in the original preparation. NGF preparations were purified by affinity column chromatography. The column bed consisted of purified murine monoclonal IgG antibody to 2.5S NGF (clone β1, kindly provided by E. M. Shooter, Stanford University) covalently attached to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The 2.5S NGF-rich starting material in 0.02 M sodium phosphate, pH 7.2/0.14 M NaCl (PBS) was equilibrated and applied to the column at 4°C and washed with PBS until the A_{280} returned to baseline. The retained 2.5S NGF was eluted with 0.1 M glycine·HCl buffer with 1.0 M Tris·HCl, pH 7.5, dialyzed against PBS, divided into aliquots, and stored at −70°C. This preparation on polyacrylamide gel electrophoresis (PAGE) showed a single protein band at 27 kDa. Electrophoresis of the 2.5S NGF at 1 mg/ml in a 16% polyacrylamide gel containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$/PAGE) showed the only stained bands (silver stain) to be the expected doublet at 13.5 kDa, with some minor diffuse staining at the buffer front (probably degradation products). This material was further characterized by ^{125}I-labeled rabbit polyclonal anti-NGF and a murine monoclonal anti-NGF by immunoprecipitation after electroblotting (see below). The 2.5S NGF affinity-purified preparation eluted as a single protein peak on a TSK 3000 HPLC column (Beckman) with a retention time that corresponded to 27 kDa (2.5S NGF dimer).

Neurotropic biological activity was measured in the dissociated cell assay, using neonatal mouse superior cervical ganglion neurons (28, 29); half-maximal response was at 1 ng/ml. The 7S form of NGF was prepared according to a modification of the procedure of Varon *et al.* (25); it also had half-maximal activity in the dissociated cell assay at 1 ng/ml.

Preparation and Purification of NGF Antisera. For the preparation of 2.5S NGF antiserum, New Zealand White rabbits received an initial injection of 0.5 mg of NGF in PBS mixed with 2 vol of Freund's complete adjuvant and injected intradermally in 20 to 30 sites. After 4 weeks, rabbits were boosted with 0.1 mg of 2.5S NGF in Freund's complete adjuvant, intradermally at multiple sites. Thereafter, monthly boosts of 0.1 mg of 2.5S NGF were made in Freund's incomplete adjuvant. Blood was collected from the ear veins, beginning 10 days after the second boost. Blood was allowed to clot at room temperature, and serum was collected, heat inactivated at 56°C for 30 min, cooled, and sterilized by filtration. To determine serum titers of anti-NGF, serial dilutions of the antiserum in culture medium were combined with equal volumes of medium containing 7S NGF at 20 ng/ml (final concentration 10 ng/ml), incubated at room temperature for 1 hr, and assayed for neutralizing activity by the dissociated cell method (28, 30). A 10,000-fold dilution of antiserum completely blocked the activity of 7S NGF at 10 ng/ml.

The specificity of the antiserum and purity of the NGF preparations were determined by Ouchterlony double diffusion analysis and by immunoblotting. Antiserum to NGF gave single precipitin lines with the crude salivary gland homogenate and with the post-acid elution fraction as well as with purified 7S and 2.5S NGF. For immunoblots, the IgG fraction of either the rabbit antiserum or β1 murine monoclonal anti-2.5S NGF was isolated by precipitation with ammonium sulfate or staphylococcal protein A and labeled with ^{125}I. NGF preparations were separated by electrophoresis on 16% polyacrylamide gels in the presence of $NaDodSO_4$ as above, transferred to nitrocellulose paper, incubated with labeled polyclonal and monoclonal anti-NGF (28, 30), and subjected to autoradiography. The only bands labeled by either anti-NGF were the 13.5-kDa doublets in lanes corresponding to 2.5S NGF, 7S NGF, and the salivary gland homogenate. No antibodies bound to mouse serum proteins, renin (10 μg), or epidermal growth factor (10 μg) run in parallel.

NGF Used in Colony Assays. Various concentrations of 2.5S NGF (5–500 ng/ml) were added to the methylcellulose cultures and CSA was determined. The monoclonal antibody was used to further purify NGF after the last column step, at which point it had already been shown to be pure by immunoblotting (above). The NGF was eluted with 0.1 M glycine·HCl buffer, and the eluate was concentrated and dialyzed against PBS. This material was highly active in the neurite outgrowth assay (26, 31).

RESULTS

CSA of 2.5S NGF Preparation. As shown in Fig. 1, NGF stimulated a dose-dependent increase in colonies, up to a mean total number of 19 colonies per 10^6 NAMC plated when used at concentrations between 5 and 500 ng/ml. Since the Mo T-cell line or lectin-stimulated T cells are known to produce colony-stimulating factors, including basophil/eosinophil growth-promoting activities (12–18, 29, 32), Mo-CM was used as a positive control; a mean total number of 35 colonies grew in the presence of 5% (vol/vol) Mo-CM. Although the maximal CSA of NGF (500 ng/ml) was about half that of Mo-CM, NGF augmented the effect of Mo-CM. The addition of NGF to NAMC in cultures with or without Mo-CM stimulated dose-dependent growth of both Eo-type and GM-type colonies but not of M-type colonies (Fig. 1). In addition, NGF significantly increased the number of basophilic-cell-containing colonies at concentrations of 50 and 500 ng/ml as well as the proportion of basophilic cells in Eo-type

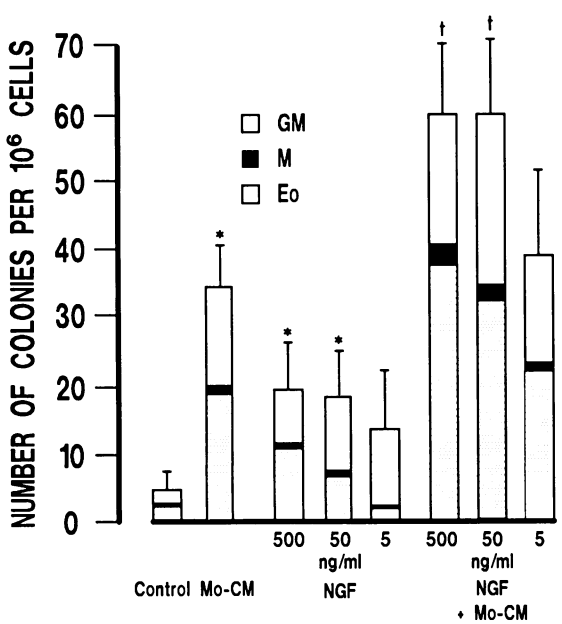

FIG. 1. CSA of 2.5S NGF. Various concentrations of 2.5S NGF were added to 1 × 10^6 NAMC in methylcellulose cultures with or without 5% (vol/vol) Mo-CM, and colonies were counted at 14 days. Colony numbers are expressed as means of seven separate experiments and vertical bars indicate SEM of total colony numbers.
*Significantly higher than control at $P < 0.05$ by paired Student's *t* test.
†Significantly higher than Mo-CM at $P < 0.05$.

Table 1. Development of basophil/mast cell colonies induced by 2.5S NGF

Condition	Mo-CM	No. of basophil/mast cell colonies per 10^6 cells
Medium control	−	0.2 ± 0.1
Positive control	+	4.2 ± 1.3*
2.5S NGF, ng/ml		
500	−	5.9 ± 2.6*
50	−	5.4 ± 1.3*
5	−	2.0 ± 0.9
500	+	17.3 ± 4.7†
50	+	11.9 ± 2.5†
5	+	6.8 ± 1.4

A basophil/mast cell colony is defined as one containing ≥5% basophilic or metachromatic cells (see text). Mo-CM (5%, vol/vol) was present (+) or absent (−) as indicated. Values are expressed as means ± SEM of five separate experiments.
*$P < 0.05$, compared to medium control by Student's t test.
†$P < 0.05$, compared to positive control.

but not GM- or M-type colonies (Table 1). Basophilic-cell CSA, defined as activity promoting basophil/mast cell colonies (16, 18), was increased by the addition of NGF to Mo-CM.

Specificity of NGF Effect on Colony Growth. The specificity of the NGF-mediated colony growth was examined by the use of both polyclonal and monoclonal antisera to 2.5S NGF, both of which completely blocked the biological nerve-stimulating activity of NGF (28, 30). As shown in Fig. 2, a polyclonal antiserum at a dilution of 1:20 completely blocked CSA of 2.5S NGF at 500 ng/ml. An identical dose–response curve was obtained for monoclonal anti-2.5S NGF. In a single experiment (data not shown), affinity-purified NGF had the same CSA as described for the 2.5S preparation above. Accordingly, the rest of the experiments were performed with the 2.5S NGF preparation.

Effect of T Lymphocytes on NGF-Mediated Colony Growth. We next examined the possible involvement of T cells in the potentiating effect of NGF on colony growth. Separated populations of non-T or T cells were prepared from NAMC

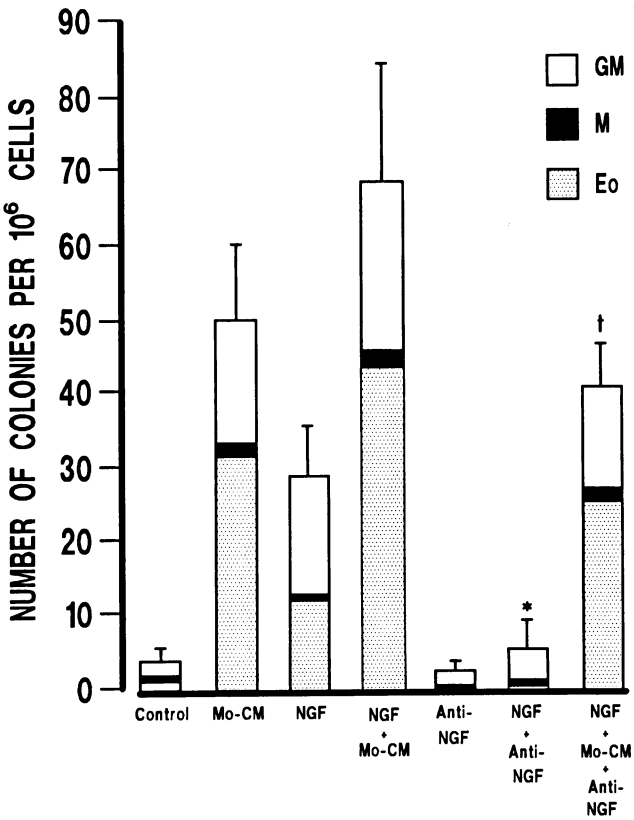

FIG. 2. Specificity of NGF-mediated CSA. Rabbit polyclonal antiserum to 2.5S NGF was added at a 1:20 dilution to 1 × 10^6 NAMC in methylcellulose cultures with or without 2.5S NGF at 500 ng/ml or Mo-CM at 5% (vol/vol) and colonies were counted on day 14 of culture. Colony numbers are expressed as means of five separate experiments and vertical bars indicate SEM of total colony numbers.
*Significantly lower than NGF at $P < 0.05$ by Student's t test.
†Significantly lower than NGF + Mo-CM at $P < 0.05$.

Table 2. T-cell-dependent CSA of 2.5S NGF

Condition	Cells	Mo-CM	NGF	No. of colonies per 10^6 cells GM	M	Eo	Total
I	1 × 10^6 unfractionated	−	−	2.3 ± 1.6	0	1.2 ± 0.8	3.5 ± 2.4
		+	−	14.0 ± 4.6*	1.5 ± 1.0	21.5 ± 3.0*	37.0 ± 2.6*
		−	+	10.9 ± 3.1*	0.1 ± 0.2	7.6 ± 3.0	18.6 ± 5.3*
		+	+	18.5 ± 4.7	1.8 ± 0.9	32.0 ± 1.5†	52.3 ± 4.4†
II	1 × 10^5 non-T	−	−	0	0	0	0
		+	−	7.2 ± 2.5	1.2 ± 0.4	8.8 ± 1.6	17.2 ± 4.4
		−	+	0.3 ± 0.2	0	0	0.3 ± 0.2
		+	+	8.5 ± 3.0	0.8 ± 0.4	9.5 ± 1.6	18.8 ± 4.6
III	1 × 10^6 T	−	−	0	0	0	0
		+	−	0.8 ± 0.4	0	0.7 ± 0.3	1.5 ± 0.7
		−	+	0	0	0	0
		+	+	0	0	0.5 ± 0.4	0.5 ± 0.4
IV	1 × 10^5 non-T + 1 × 10^6 T	−	−	0	0	0	0
		+	−	10.3 ± 1.3	0.2 ± 0.1	22.7 ± 2.1	33.2 ± 0.9
		−	+	5.3 ± 1.3	0.1 ± 0.1	1.8 ± 0.5	7.2 ± 1.7
		+	+	16.5 ± 1.9†	0.8 ± 0.2†	26.0 ± 0.9	43.5 ± 0.9†

Mo-CM was present at 5% (vol/vol) (+) or absent (−) as indicated. NGF was present at 500 ng/ml (+) or absent (−). Values are expressed as means ± SEM of five separate experiments. All statistical tests were done by analysis of variance and paired t tests (two-tailed). For all cell populations except − Mo-CM and − NGF, condition IV differed significantly from condition II, $P < 0.02$; conditions II and IV differed from condition III, $P < 0.01$.
*$P < 0.05$, compared to − Mo-CM and − NGF for same cell population.
†$P < 0.05$, compared to + Mo-CM and − NGF for same cell population.

by using rosetting with sheep erythrocytes treated with 2-aminoacetylisothiouronium bromide hydrobromide (Sigma) (33, 34). Using this method, we obtained a purity of 72–92% T cells in our NAMC preparations. When 1×10^5 non-T cells alone or 1×10^6 T cells alone were cultured in the presence of NGF at 500 ng/ml, no CSA was detected in the presence or absence of Mo-CM (Table 2). CSA of NGF preparations was observed only when non-T- and T-cell fractions were mixed. Thus, granulocyte colony-forming cells were contained in the non-T-cell fraction and could not be stimulated by NGF to grow in the absence of T cells.

DISCUSSION

The results obtained clearly demonstrate that, under the conditions tested, murine NGF induces the differentiation of neutrophils/macrophages, eosinophils, and basophilic cells from their progenitors in human peripheral blood. Moreover, the CSA of NGF was observed only in the presence of unfractionated or sheep-erythrocyte-rosette-enriched cells, suggesting that NGF may stimulate secretion of hemopoietic growth factors from T cells, which are known to synthesize factors capable of inducing the differentiation of eosinophils, basophils, and mast cells (12–18, 29, 32, 35–41). Since a significant increase in Eo-type and, specifically, in basophil/mast cell colonies developed in response to NGF, NGF may activate T cells to locally secrete hemopoietic factors at sites of inflammation and thus induce differentiation of eosinophils and basophils/mast cells from recently migrated blood-borne progenitors (14–18). Similar interactions among T cells, endothelial cells, fibroblasts, and monocytes leading to increased production of CSA have been shown to occur (42, 43).

Mast cells are involved in many types of inflammation and repair processes and are found in increased numbers in scars and fibrotic tissue in skin, intestine, and lung (44–48). Increased mast cell numbers are found in callus formation in repairing bone fractures and in pathological states as inflammatory bowel disease and scleroderma (46–49). In addition to their involvement in immediate and delayed hypersensitivity reactions (50), mast cells may be involved in tissue repair processes.

Our previous work suggesting the elaboration of nasal mucosal basophil/mast cell growth-promoting factors in nasal allergy (14–18) supports the concept that, *in situ*, growth and differentiation of hemopoietic cells are involved in allergic inflammatory disease. NGF is secreted by fibroblasts (46) and can induce in dose-dependent manner significant increases in mast cell numbers of both mucosal and connective tissue type, according to their normal predominance in a particular tissue, after injection into neonatal rats (8, 9). Since NGF can also promote the effect of T-cell-derived lymphokines or induce T cells to secrete CSA, NGF may play a significant role in both inflammation and tissue repair, as has already been suggested in wound-healing experiments (2, 7). Indeed, NGF has been shown to possess a potent anti-inflammatory activity (2) and may thus form part of a family of peptide growth factors such as platelet-derived growth factor and transforming growth factor (TGF-α and TGF-β), which are both produced in inflammation and play putative roles in both inflammation and tissue repair. Recently, interleukin 1 has been shown to induce granulocyte (G-) and granulocyte-macrophage (GM-) colony-stimulating factor (CSF) production by fibroblasts and endothelial cells (42, 43, 51, 52), and tumor necrosis factor α has also been found to induce both G-CSF and GM-CSF (53–55), lending credence to the concept that micro-environmental (*in situ*) hemopoietic processes are of fundamental importance in tissue inflammation (15, 17, 52, 55).

With regard to the selectivity of the NGF effect in hemopoiesis shown here, it is clear that other molecules with similar charge or biological effects such as epidermal growth factor, insulin, cytochrome *c*, and other proteins do not cause mast cell hyperplasia *in vivo* (3, 8). Moreover, under serum-free conditions, hemopoietic progenitors are not stimulated to grow or differentiate in the presence of transferrin, bovine serum albumin, insulin, and lipids (56). Nonetheless, it is possible that other peptide growth factors such as epidermal growth factor or fibroblast growth factor may play roles similar to the one we have demonstrated for NGF in these studies or to the ones recently observed for interleukin 1, transforming growth factor α, or tumor necrosis factor α (42, 43, 51–55).

There is already precedent for hemopoietic growth factors derived from T cells, endothelial cells, or fibroblasts to also possess the property of functional activation of progeny (e.g., neutrophils, eosinophils) of progenitors (57–59). The stimulatory effects of murine NGF on human hemopoiesis in dose ranges similar to those in which biological activities of other growth factors and hemopoietic cytokines are demonstrable (53–55, 57–60), may represent an important biologic mechanism for such activation processes and stand in contrast to the inhibitory effects recently reported for tumor necrosis factor β (54, 60).

The experiments described here present evidence for an important additional role for NGF. The observations imply the presence of NGF receptors on some T cells (61) and confirm that, in the human, NGF can act to promote differentiation of specific granulocytes such as basophils/mast cells and eosinophils. These observations raise the possibility of even broader biological actions of this neurotropic peptide on immune and nonimmune responses.

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