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Nerve growth factor induces growth and differentiation of human B lymphocytes

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ABSTRACT **Nerve growth factor (NGF) is known to affect peripheral sympathetic and sensory neurons as well as defined populations of neurons in the central nervous system. This paper presents evidence that NGF is also active in modulation of B-cell-mediated immune responses. NGF receptors were immunoprecipitated from highly purified human B-cell populations, and to a lesser extent, from T-cell populations, by using a monoclonal antibody recognizing NGF receptors present on neural cells. NGF receptors were also detected in significant amounts in human spleen and lymph node tissue. In addition, NGF induced a dose-dependent increase in B-cell DNA synthesis as determined by incorporation of [^{3}H]thymidine. This B-cell growth-promoting activity was inhibited by a neutralizing anti-NGF monoclonal antibody. Immunoglobulin secretion, principally affecting IgM synthesis, was also modulated by NGF. The concentrations that affected B-cell proliferation are consistent with the presence of functional high-affinity NGF receptors. The results suggest that NGF, in addition to its neurotrophic function, also acts as an immunoregulatory cytokine.**

Nerve growth factor (NGF) is a well-characterized neurotrophic protein essential for development and differentiation of peripheral sympathetic neurons and the majority of neural crest-derived sensory nerve cells (1–3). In addition to its peripheral actions, NGF may play a physiological role in the mammalian central nervous system as a trophic agent for basal forebrain cholinergic neurons (4, 5). The biological actions of NGF on responsive neurons are mediated by interaction with a specific cell membrane-bound receptor. The NGF–receptor complex is internalized and retrogradely axonally transported from nerve terminals to neuronal cell bodies where specific cellular processes are mediated (6).

In addition to its neurotrophic actions, there is increasing evidence that the NGF/NGF receptor system may have broader physiological effects than regulating neuronal differentiation. Experimental evidence suggests that NGF may modulate inflammatory responses. For example, NGF induces a rapid change in the shape of blood platelets (7). It increases the number of mast cells in neonatal rats (8), leads to a massive degranulation of rat peritoneal mast cells (9–11), promotes differentiation of specific granulocytes (12), and stimulates wound healing (13). Moreover, biologically active NGF enhances vascular permeability in the skin in a dose-dependent fashion (14), an observation suggesting that NGF is involved in acute inflammatory responses. Furthermore, treatment of young rats with NGF prior to and after immunization with sheep erythrocytes results in an enhancement of T-lymphocyte-dependent antibody synthesis (15), suggesting that NGF participates in immune responses. Studies on the regulatory mechanisms involved in NGF production reveal that inflammatory stimuli contribute significantly to an increased NGF production (16). Interleukin 1 (IL-1), a mediator of inflammation and tissue degradation (17), is a potent inducer of NGF synthesis in the rat sciatic nerve (18) and in the central nervous system (19). Moreover, IL-1 (20) and B-cell stimulatory factor 2 (IL-6) (21) induce increased production and secretion of biologically active NGF by astrocytes. Functional receptors for NGF have been found on rat spleen mononuclear cells (22, 23), suggesting that NGF may be involved in the immune response.

By using a monoclonal antibody against human NGF receptor, an extremely sensitive, specific immunoprecipitation assay for the quantitation of human NGF receptors has been established (24). Our studies on human lymphocytes reveal that both T and B lymphocytes express functional NGF receptors.

MATERIALS AND METHODS

Monocyte and Lymphocyte Preparations. Human peripheral blood mononuclear cells were isolated from blood on Ficoll-Paque gradients (Pharmacia). The mononuclear cells, suspended in 20 ml of phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}) supplemented with 0.2% bovine serum albumin (fraction V; Calbiochem), were loaded into a Beckman elutriation centrifuge (J-6M, JE-6B rotor, standard chamber, all from Beckman). Separation by counterflow centrifugal elutriation was performed at a constant rotor speed (2000 rpm) and variable flow rate (6–18 ml/min). Cells were collected in 50-ml fractions and cell size distribution was analyzed with a microcell counter CC-130 (Sysmex; Toa Medical Electronics, Kobe, Japan) interfaced with an Apple IIe computer. The lymphocyte population was eluted at flow rates of 12–14 ml/min, whereas the monocyte population was eluted at flow rates of 17–18 ml/min. The purity of the monocyte and lymphocyte preparations has been described (25). The lymphocytes were further separated into T- and B-cell populations based on rosette formation using magnetic beads coated with anti-CD19 monoclonal antibody (26). The positively selected B cells were more than 98% $CD20^+$, $HLA\text{-}DR^+$, and surface Ig^+. Fewer than 1% $CD3^+$, $CD4^+$, or $CD8^+$ cells were detected in the B-cell preparation. In contrast, the negatively selected T-cell population contained fewer than 1% $CD20^+$, $HLA\text{-}DR^+$, or surface Ig^+ cells. More than 90% were $CD3^+$, 50–70% were $CD4^+$, and 20–40% were $CD8^+$. The purified cell populations were centrifuged and resuspended in culture medium, RPMI 1640 (GIBCO) supplemented with 5% fetal bovine serum (FBS) (Amimed, Basel), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acid solution, 10 mM Hepes, 100 units of penicillin per ml, and 100 μg of streptomycin per ml (all from GIBCO) (RPMI/5% FBS).

ELISA Assay for Immunoglobulin Subtypes. Culture supernatants (100 μl) from treated B cells were collected after 5 days and assayed for immunoglobulin content by a solid-phase ELISA (27). In brief, microtitration plates were coated

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Abbreviations: NGF, nerve growth factor; IL-*n*, interleukin *n*.

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with mouse monoclonal anti-human heavy chain-specific antibodies (Litton Bionetics) for 18 hr at 4°C. After the plates were washed with PBS/0.05% Tween 20 (Merck), nonspecific binding sites were blocked by incubation with 0.5% bovine serum albumin for 30 min at 20°C. B-cell supernatants were incubated 2 hr at 20°C. The binding of secreted immunoglobulins was detected by incubation for 2 hr at 20°C with a peroxidase-conjugated mouse monoclonal anti-human antibody (Hybritech). Peroxidase development was read at 492 nm on an ELISA plate reader (Flow Laboratories). Purified myeloma proteins of each isotype (Milan Analytica, La Roche) were used as references. The optical densities obtained from the B-cell supernatants were fitted with titration standards of reference immunoglobulins, allowing calculation of immunoglobulin concentrations. The sensitivities of the assays were 10 ng/ml, 30 ng/ml, and 30 ng/ml for IgG, IgM, and IgA, respectively.

Proliferation Assay. T or B cells were cultured in triplicate microtiter wells at 3×10^4 cells per well for 5 days (37°C, 5% CO_2) in medium with various concentrations of NGF. In experiments using anti-NGF neutralizing antibodies, lymphocyte cultures were established as above, with or without the addition of monoclonal anti-NGF (50 μg/ml) or an equal concentration of an isotype-matched control antibody. Following incubation, cultures were incubated with [*methyl*-^{3}H]thymidine [Radiochemical Centre; 1 μCi (37 kBq) per well] for the last 6 hr of culture. The means and standard errors of the mean (SEM) of incorporated [^{3}H]thymidine were determined by liquid scintillation counting after cells were collected on glass-fiber filters (28).

NGF Receptor Assay. NGF receptor molecules were quantified with an immunoprecipitation assay (24) using a monoclonal antibody directed against human NGF receptor [hybridoma clone 200-3-G6-4 (20.4 IgG) developed by Ross *et al.* (29) and purchased from the American Type Culture Collection]. In brief, steady-state binding of ^{125}I-labeled NGF (2 nM) was assayed with cells or with membrane fractions prepared by the method of Costrini and Bradshaw (30). A 1000-fold excess of unlabeled NGF was included in parallel tubes to determine nonspecific binding. After covalent attachment of the ^{125}I-NGF to the receptor by addition of 20 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce), the labeled complex was solubilized for 60 min with 2% octyl β-D-glucoside (Sigma) and further incubated for 90 min with 10 μl of 20.4 IgG antibody solution. Rabbit anti-mouse IgG-precoated protein A-Sepharose CL-4B (Pharmacia) was then added to the reaction mixture to immunoprecipitate the ^{125}I-NGF–receptor complexes. These complexes were then quantified by layering aliquots of protein A-Sepharose suspension onto 250 μl of 0.15 M sucrose in 400 μl microcentrifuge tubes. After centrifugation (12,000 × *g*, 1 min) tips containing the complexes were cut from the frozen tubes and the amount of radioactivity in the pellet was determined in a γ counter. Background nonspecific values, which were always <40% of total binding values, were subtracted for correction. In some cases, samples were further analyzed by SDS/7% PAGE (31), followed by autoradiography using Kodak X-Omat AR film with DuPont Lightning Plus intensifying screens.

Preparation of NGF and ^{125}I-NGF. Mouse NGF (2.5S form) was isolated from adult male mouse submandibular glands by the method of Bocchini and Angeletti (32) with the modifications described by Weskamp and Otten (16). High-pressure liquid chromatography (HPLC) purification of NGF was performed as described (33). NGF (2.5S) was radioiodinated by using immobilized glucose oxidase and lactoperoxidase (Enzymobeads; Bio-Rad) as described by the manufacturer. ^{125}I-NGF was separated from nonincorporated ^{125}I by gel filtration on a column (0.75 × 30 cm) of Sephadex G-25 fine (Pharmacia) equilibrated with PBS (pH 7.4) containing 0.1% bovine serum albumin. Fractions collected at the void volume were 94–97% trichloroacetic acid-precipitable and migrated to a position identical to noniodinated NGF in SDS/polyacrylamide gels. The specific activities of the ^{125}I-NGF preparations ranged from 2500 to 4000 cpm/fmol. ^{125}I-NGF was stored at 4°C and used within 2 weeks of preparation.

Human Tissues. Human tissues (spleen, lymph nodes) were kindly provided by F. Gudat, J. Ulrich, and A. Probst (Institute of Pathology, University of Basel). Human blood was obtained from healthy donors.

RESULTS

Detection of NGF Receptors in Human Spleen, Lymph Nodes, and Mononuclear Cells. Immunoprecipitation of the NGF receptor in spleen and lymph node tissue revealed a broad band at ≈85 kDa (Fig. 1*A*). A similar molecular mass for the NGF receptor has been reported (29). Binding of ^{125}I-NGF to the receptor was specific, as incubation in the presence of 1000-fold excess unlabeled NGF protein (2 μM) reduced total binding by 80–85% (Fig. 1*B*).

Estimation of NGF receptors in blood cells from blood of healthy volunteers showed that B lymphocytes contained more NGF receptor protein than T lymphocytes (Table 1). Monocytes expressed detectable amounts, whereas no specific receptor protein was found in erythrocytes (Table 1). In comparison to neurons, levels of NGF receptors on lymphocytes are lower by a factor of 500.

Effect of NGF on T- and B-Cell Proliferation. To assess whether NGF binding sites expressed by lymphocytes are functional NGF receptors, the effects of NGF on B- and T-lymphocyte proliferation were studied. Fig. 2 shows representative results derived from a series of three independent experiments using blood of three different donors. [^{3}H]Thymidine incorporation into lymphocytes was monitored in the

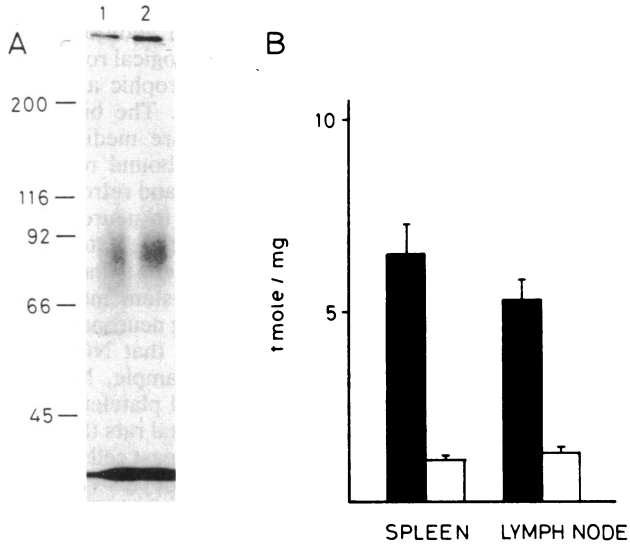

FIG. 1. NGF receptor in human spleen and lymph nodes. Plasma membrane-enriched fractions (0.5 mg of protein per assay) were incubated with 2 nM ^{125}I-NGF in either the absence or the presence of a 1000-fold excess of unlabeled NGF. After crosslinking and solubilization, the extracts were immunoprecipitated with the monoclonal antibody 20.4 IgG. (*A*) Analysis of the immunoprecipitates by SDS/7% PAGE followed by autoradiography. Lane 1, spleen; lane 2, lymph node. (*B*) NGF receptor content of spleen and lymph nodes expressed in femtomoles per milligram of protein. Data are mean ± SEM values from three independent determinations, each done in duplicate. Solid bars, specific binding; open bars, nonspecific binding. Statistical analysis, performed with Student's *t* test, showed that specific binding was significantly ($P < 0.001$) greater than nonspecific binding for both tissues.

Table 1. NGF receptor expression of human blood cell populations

Cells	NGF receptor, fmol per 10^8 cells
B lymphocytes	3.94 ± 0.63
T lymphocytes	2.76 ± 0.30
Monocytes	1.06 ± 0.19
Erythrocytes	<0.1

Values are means ± SEM of independent determinations, each done in duplicate and performed with blood cells of six different donors.

presence of NGF with or without specific monoclonal anti-NGF antibodies known to completely block the biological action of NGF on neuronal cells (16).

In B cells (Fig. 2 *Upper*) a detectable, significant ($P < 0.05$) growth response was elicited with as little as 0.1 ng (4 fmol) of NGF per ml. A third-order linear regression analysis determined the EC_{50} of NGF on B cells to be 0.6 ng/ml, suggesting that a high-affinity NGF receptor ($K_d \approx 20$ pM) is involved in NGF-mediated B-cell growth. The specificity of the NGF effect on B-cell growth is documented by the finding that addition of an excess of monoclonal anti-NGF antibody almost completely abolished NGF-induced stimulation. An isotype-matched control antibody did not affect NGF-induced lymphocyte proliferation (data not shown).

In contrast to B lymphocytes, T-cell proliferation was slightly (but significantly, $P < 0.05$) increased at the highest concentrations of NGF (Fig. 2 *Lower*).

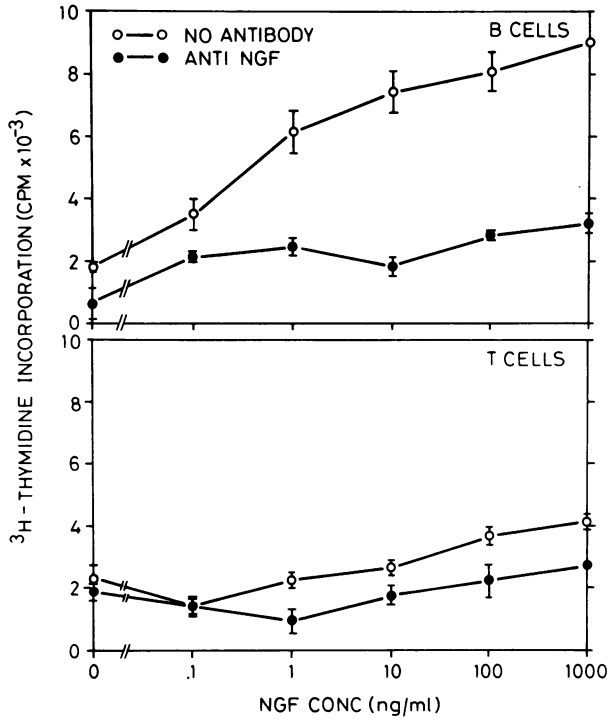

FIG. 2. Effects of NGF on DNA synthesis of B cells (*Upper*) and T cells (*Lower*). Lymphocytes were cultured at 3×10^4 cells per well in the presence of medium (0 value on abscissa) or medium plus the indicated concentrations of NGF, with (●) or without (○) anti-NGF neutralizing monoclonal antibody (50 μg/ml). After incubation for 5 days, cells were incubated for 6 hr with [^{3}H]thymidine (1 μCi per well). The data are presented as mean ± SEM for incorporated thymidine, calculated from triplicate samples in a single representative experiment performed with the leukocytes of an individual blood donor. Statistically significant differences ($P < 0.05$) between NGF alone and NGF with anti-NGF antibody were determined at all NGF concentrations ≥0.1 ng/ml in B-cell cultures. In T cell cultures, significant inhibition by antibody was observed with NGF at 100 and 1000 ng/ml.

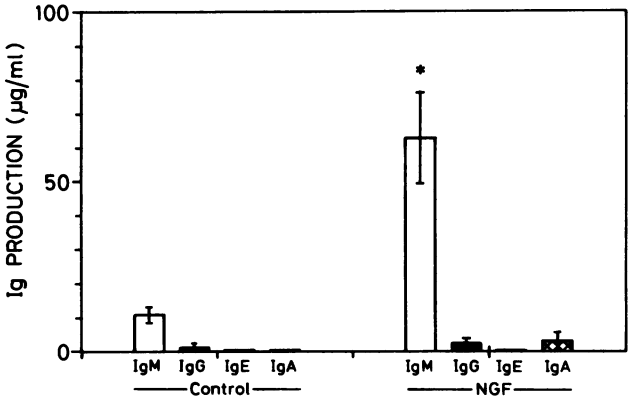

FIG. 3. Secretion of immunoglobulins of each isotype by B cells. B cells were cultured as in Fig. 2 for 5 days in medium (control) or in the presence of NGF (100 ng/ml). Supernatant was removed and assayed by ELISA using monoclonal antibodies specific for each isotype. The data are presented as mean ± SEM for immunoglobulin concentrations obtained by fitting optical densities generated from the ELISA to standard curves for each isotype. The results were calculated from triplicate samples in a single representative experiment. Asterisk indicates a difference from untreated controls ($P < 0.001$).

Effect of NGF on Immunoglobulin Secretion. Determinations were made of immunoglobulin subtypes secreted into supernatants of cultured B cells after 5 days in either the absence or the presence of NGF (100 ng/ml). Determination of immunoglobulin synthesis was performed with blood from three donors (Fig. 3). Control human B cells produced about 10 μg of IgM per ml, extremely low levels of IgG, and no detectable IgE or IgA. However, stimulation of B cells with NGF resulted in an ≈6-fold increase ($P < 0.001$) in IgM but little change in IgG or IgE. Levels of IgA were slightly (but significantly, $P < 0.05$) increased. This stimulatory effect was dose-dependent (Fig. 4). Half-maximal stimulation was obtained with NGF at 5 ng/ml and a maximal effect was consistently elicited at 100 ng/ml. Dose-dependent stimulation of IgM secretion required higher NGF concentrations than does NGF-mediated B-cell proliferation. However, the sensitivity of the ELISA assays used for determination of immunoglobulin secretion is less than that of the proliferation assay, making estimations of NGF receptor affinity difficult to interpret.

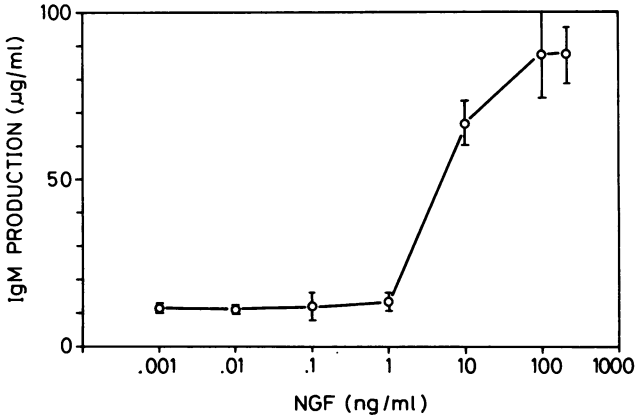

FIG. 4. Secretion of IgM by B cells. B cells were cultured as in Fig. 2 for 5 days in the presence of the indicated concentrations of NGF. Supernatant was removed and assayed for IgM by ELISA. The data are presented as mean ± SEM for IgM concentrations obtained by fitting optical densities generated from ELISA to an IgM standard curve. The results were calculated from triplicate samples in a single representative experiment.

DISCUSSION

This report provides evidence that human lymphocytes express functional NGF receptors. The use of a sensitive immunoprecipitation assay for the quantitation of NGF binding sites allowed detection of NGF receptors on B and T lymphocytes. Interaction of NGF with these receptors induced a proliferative response in human B and T cells, as well as differentiation of B cells into antibody-secreting cells. NGF promoted IgM secretion, and to a lesser extent IgA secretion, but failed to stimulate secretion of IgG or IgE. These observations suggest that NGF regulates both growth and differentiation of B cells.

The use of highly purified B cells minimized the possibility that the observed NGF effects were indirect—i.e., the result of factors induced from a minor population of contaminating cells. Similarly, the 2.5S NGF preparation itself was free of detectable contamination (yielding a single band in SDS/polyacrylamide gels), and HPLC-purified β-NGF was found to elicit similar responses. The observation that a neutralizing monoclonal antibody specific for NGF can block NGF-induced B-cell responses strongly indicates that NGF mediates these effects directly. NGF induced B-cell proliferation ([^{3}H]thymidine incorporation) at concentrations of 0.1–10 ng/ml (Fig. 2) consistent with the activity of a high-affinity receptor (34, 35).

A variety of cytokines have been described that act upon B-cell growth (36, 37) and differentiation (38). Similarly, cytokines that preferentially select particular immunoglobulin isotypes have been demonstrated (39). The best documented of these include IL-2, IL-4, IL-5, IL-6, and γ-interferon. The present demonstration of functional NGF receptors on B cells implies that NGF may act as an immunomodulatory cytokine, mediating interactions between cells of the nervous and immune systems. The B-cell surface marker CDw40 has recently been found to have extensive structural homology to the NGF receptor (40). This result suggests that a NGF-like molecule could play a role in B-cell regulation. The present data strongly indicate that NGF itself has such immunoregulatory activities. However, it appears unlikely that NGF effects on B cells are necessarily mediated by binding to CDw40. Immunohistochemical analysis of NGF receptor and CDw40 expression by the use of specific antibodies revealed a clear difference in staining patterns in B-cell-rich areas of human lymph nodes (F. Gudat and U.O. unpublished observation), suggesting that these molecules are present on different B-cell subpopulations. That two structurally related cell surface molecules such as the NGF receptor and CDw40 trigger similar responses in B cells suggests that there is an interaction between these two molecules, at least at the level of intracellular signaling (41), that could be an important mechanism in B-cell regulation.

To date, relatively little is known about the molecular mechanism regulating expression of NGF and its receptor. Studies of NGF synthesis have demonstrated that inflammatory stimuli significantly increase NGF production (16). IL-1 enhances the expression of NGF mRNA in peripheral tissues (18), as well as in brain following intracerebral injection (19). Moreover, IL-6 enhances NGF release by astrocytes (21). Therefore it appears likely that cytokines such as IL-1 and IL-6, which accumulate at sites of injury and infection, induce localized NGF production, thereby influencing neuronal regeneration as well as immune responses.

There is evidence for B-lymphocyte activation in the central nervous system in viral diseases. B lymphocytes have been found in brain tissue during the course of acute or chronic viral infections and in multiple sclerosis (21, 42, 43). The signals that trigger B cells in the brain to undergo clonal expansion and maturation are not known. In an experimental model of multiple sclerosis, NGF was found to accumulate at sites of inflammation in the brain (H. P. Lorez, W. Fierz, and U.O. unpublished observation). This finding implies that local NGF not only plays a role in tissue repair after brain injury (4) but also provides the requisite signals for B-cell growth and maturation.

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