

Human CD4+ T cell clones produce and release nerve growth factor and express high-affinity nerve growth factor receptors

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Background: Increasing evidence shows that nerve growth factor (NGF) plays a role in the complex and fascinating linkage between the nervous and the immune systems due to its ability to modulate functions of several inflammatory cells.

Objective: To investigate NGF receptor expression and NGF production and release by human CD4+ cells clones, which have primary relevance in modulating inflammatory events through their different subsets of functional phenotypes.

Methods: The expression of NGF and a transmembrane tyrosine kinase (TrkA) was evaluated by immunohistochemistry and flow cytometry analysis in five T_{H0}, six T_{H1}, and five T_{H2} cell clones derived from human circulating mononuclear blood cells. Moreover, the amount of NGF protein was assessed by measuring the NGF levels in culture supernatants of the T cell clones before stimulation and 48 hours after phytohemagglutinin (PHA) activation by use of an immunochemical assay.

Results: Our data have shown that in unstimulated conditions, human CD4+ T cell clones express both immunoreactivity for NGF and the TrkA NGF receptor irrespective of their cytokine profile. Moreover, T_{H1} and T_{H2} clones, but not T_{H0} clones, secrete NGF in basal conditions. PHA activation induces NGF secretion in T_{H0} clones and a significant increase of NGF levels in T_{H2} ($p < 0.05$), but not in T_{H1} culture supernatants.

Conclusions: Results obtained represent the first evidence of TrkA expression and NGF production and release in human CD4+ cell clones and suggest a possible functional role of NGF in modulating the immune and inflammatory network. (*J Allergy Clin Immunol* 1997;100:408-14.)

Key words: Nerve growth factor, tyrosine kinase receptor, lymphocyte, CD4+ T cells, T_{H2} clones

Abbreviations used

BSS:	Buffered saline solution
INF γ :	Interferon- γ
MFI:	Mean fluorescence intensity
NGF:	Nerve growth factor
PBMCs:	Peripheral blood mononuclear cells
PHA:	Phytohemagglutinin
T _{H0} , T _{H1} , T _{H2} :	T helper cell subsets
TNF:	Tumor necrosis factor
TrkA:	Tyrosine kinase

Nerve growth factor (NGF) is the best characterized neurotrophin essential for neuron survival, differentiation, and function in the peripheral and central nervous systems.¹ Several recent studies have indicated that NGF is involved in the immune response and in the hematopoietic process.²⁻⁵ NGF increases the size and number of mast cells in peripheral tissues of neonatal rodents,⁶ and it enhances survival, phagocytosis, and superoxide production of mature murine neutrophils.⁷ It also induces mediator release by rat mast cells⁸ and human basophils.⁹ Moreover, NGF stimulates human and rat lymphocyte proliferation,^{10, 11} upregulates surface expression of the IL-2 receptor on human T and B cells,^{12, 13} and induces B cell differentiation into immunoglobulin-secreting plasma cells,¹¹ thereby stimulating the production of IgM, IgA, and IgG4.^{11, 14}

The biological effects of NGF are mediated by two classes of receptors¹⁵: p75, a 75 Kd glycoprotein belonging to a superfamily of cytokine receptors that includes tumor necrosis factor (TNF) receptor, Fas, CD27, CD40, and CD30¹⁶; and a transmembrane tyrosine kinase of 140 Kd (TrkA),¹⁷ phosphorylated on tyrosine after binding to its ligand.¹⁸ The precise role of this receptor in NGF signal transduction within the nervous system has not been fully elucidated. Several studies have indicated that stimulation of TrkA is necessary and sufficient to elicit a full biological response,^{19, 20} whereas other reports have highlighted the crucial role of the association of TrkA and

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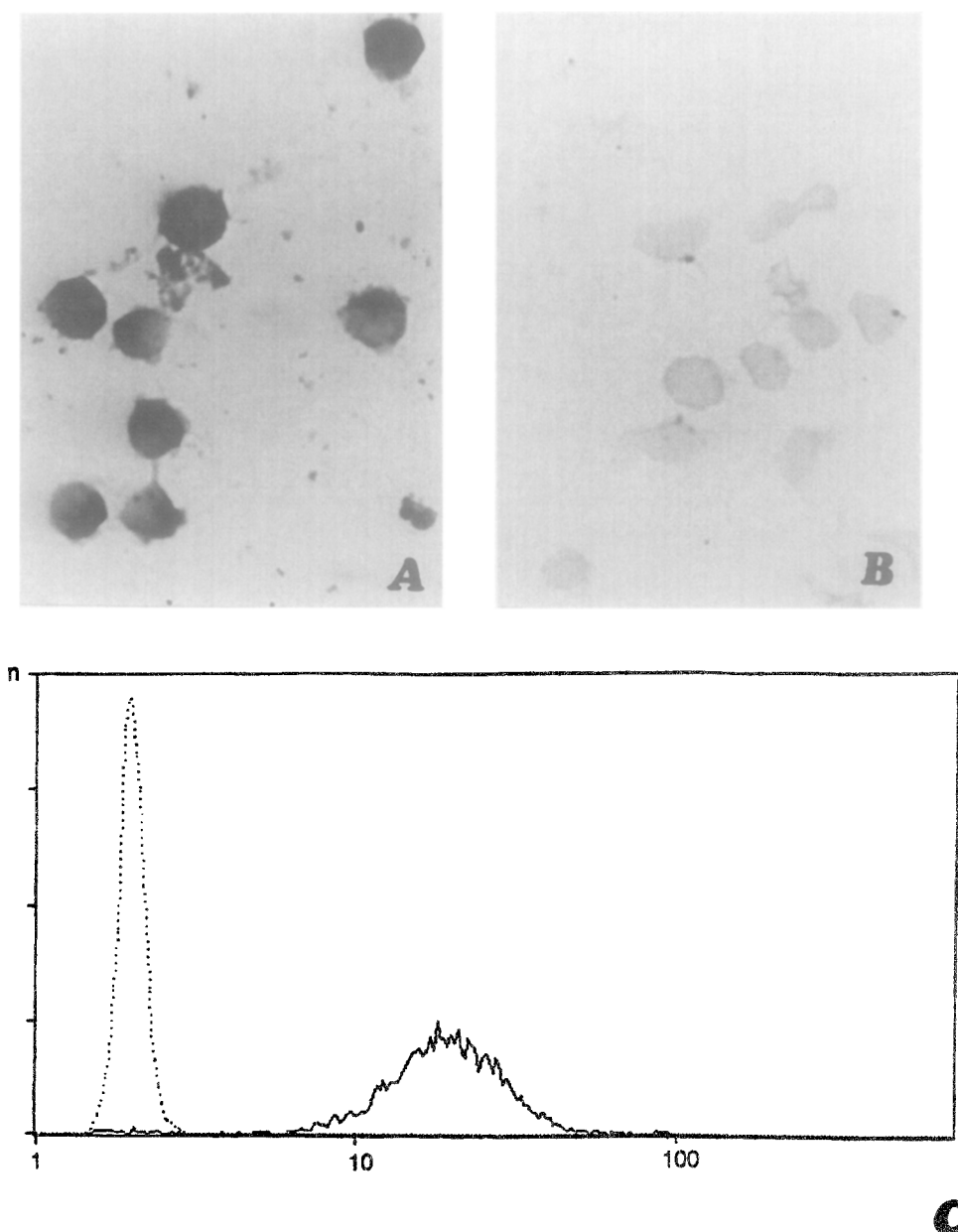


FIG. 1. Immunohistochemical TrkA pattern observed in a CTT25 clone (a T_{H2} -cell clone) (**A**) and its immunoreactivity evaluated by flow cytometric analysis (**C**). Negative controls were cells stained with nonspecific rabbit IgG (**B** and dotted line in **C**).

p75 in regulating NGF biological activities.^{21, 22} However, all of the studies seem to confirm that TrkA expression is necessary for NGF signal transduction. Recently it has been reported that activated murine T helper cell (T_H) clones express tyrosine kinase and synthesize and release NGF.²³

NGF receptors have been demonstrated on different human tissues, not only in the central and peripheral nervous systems,²⁴ but also on immune cells, including human monocytes and lymphocytes.^{11, 25, 26}

An increase of NGF levels in the blood or tissues of patients affected by different diseases (all characterized by

an activation of the immune cells) has been reported,²⁷⁻²⁹ suggesting an involvement of this molecule in immune processes. More recently, our group produced evidence that circulating NGF levels are increased in allergic diseases and asthma,³⁰ conditions characterized by a typical T_{H2} cytokine profile.³¹ NGF is produced by murine immune cells, such as mast cells³² and activated T_H clones.^{23, 33} Less consistent data are available regarding NGF production and release by human immune cells. In particular, no data are available for T_H lymphocytes, which play a key role in modulating immune reactions and promoting macrophage activation, antibody production,

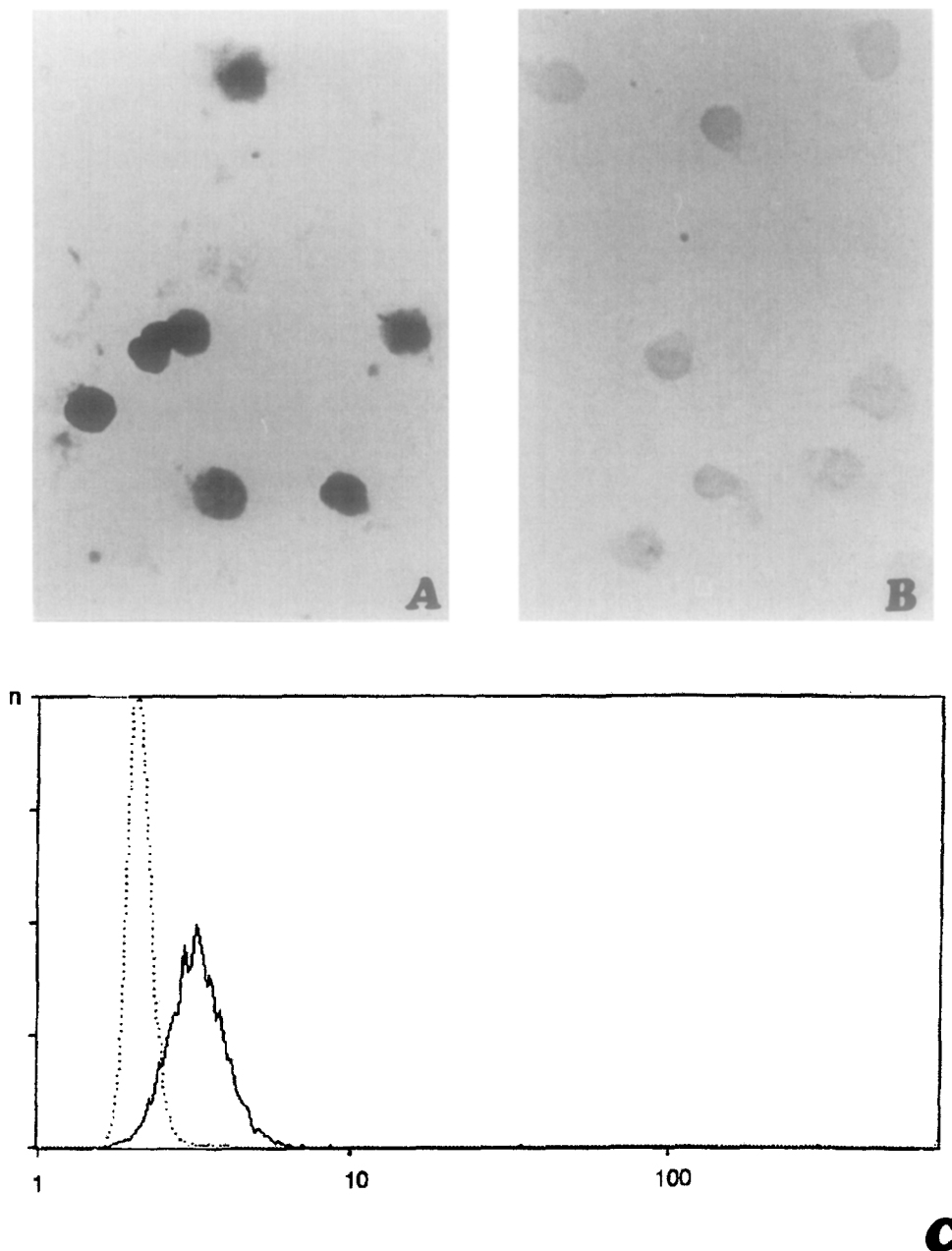


FIG. 2. Immunohistochemical NGF pattern observed in a CTT25 clone (a T_{H2} -cell clone) (A) and its immunoreactivity evaluated by flow cytometric analysis (C). Negative controls were cells stained with nonspecific goat IgG (B and dotted line in C).

mast cell growth, and eosinophil differentiation and activation. In vitro study of CD4+ T cell clones seems particularly suitable to investigate the role played by NGF in T_H -dependent immune mechanisms.

The aim of our study was to investigate if human CD4+ T cell clones with different cytokine profiles expressed NGF high-affinity receptors and if these cells produced and released this neurotrophin.

METHODS

Reagents

Purified protein derivative of *Mycobacterium tuberculosis* and tetanus toxoid were provided by the Istituto Sieroterapico e Vaccinogeno Sclavo (Siena, Italy). We purchased 70 Kd heat shock protein from StressGen Biotech Corp. (Victoria, Canada). *Toxocara canis* excretory/secretory antigen and *Dermatophagoides pteronyssinus* group I allergen (Der p I) were provided by Lofarma Allergeni (Milan, Italy).

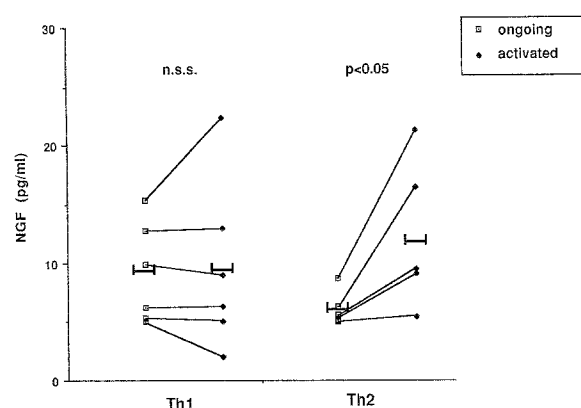


FIG. 3. NGF levels in culture supernatants of both ongoing (□) and PHA-activated (●) T_{H1} and T_{H2} clones. After activation, T_{H2} cells, but not T_{H1} cells, show a significant increase of NGF release ($p < 0.05$).

T cell cloning system

T cell clones were generated from the peripheral blood mononuclear cells (PBMCs) of five donors (four healthy subjects and one atopic subject sensitive to *D. pteronyssinus*) by limiting-dilution cloning as described previously.³⁴ Briefly, PBMCs (10^6 /ml) were stimulated for 5 days with the appropriate antigen in 2 ml RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mmol/L L-glutamine and 5% human serum. Human IL-2 (20 U/ml) was then added and cells were cultured for an additional 10 days. T cell blasts were then seeded under limiting dilution conditions (0.3 cell/well) in round-bottomed microwells containing 10^5 irradiated allogenic PBMCs (as feeder cells), phytohemagglutinin (PHA, 1% vol/vol), and IL-2 (20 U/ml). Growing T cell clones were then kept in continuous expansion by weekly addition of IL-2 (20 U/ml).

Assessment of cytokine production

To assess the cytokine profile of each clone, T cell blasts (10^6 /ml) were stimulated for 36 hours with phorbol 12-myristate 13-acetate (10 ng/ml) plus anti-CD3 antibody (100 ng/ml; Ortho Pharmaceuticals, Raritan, N.J.). Interferon- γ was measured by a commercial radioimmunoassay (Centocor Inc., Malvern, Pa.). TNF- β and IL-4 production was quantified in culture supernatants by the use of commercial ELISA (Quantikine, R&D Systems, Minneapolis, Minn.). IL-5 was measured based on the murine LyH7.B13 cell line as a source of indicator cells, as detailed elsewhere.³⁴ Six T cell clones able to produce TNF- β and IFN- γ , but not IL-4 or IL-5, were categorized as T_{H1} ; five T cell clones able to produce TNF- β , IFN- γ , IL-4, and IL-5 were categorized as T_{H0} ; and five T cell clones able to produce IL-4 and IL-5, but not TNF- β or IFN- γ , were categorized as T_{H2} clones.

Immunohistochemical analysis

Immunohistochemical analysis for NGF was performed with an affinity-purified NGF goat polyclonal antibody (1 to 0.1 μ g/ml) produced in our laboratory. Phenotypic analysis was performed by indirect immunofluorescence and immunoperoxidase according to a previously described procedure.³⁵ Immunoperoxidase and immunofluorescence localization of gp140 TrkA was also performed by use of an affinity-purified rabbit polyclonal antibody (Trk 763; Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) (0.3 μ g/ml), raised against a peptide of TrkA

TABLE I. Increase of mean fluorescence intensity relative to the control (Δ MFI) for TrkA and NGF in T cell clone subsets

Clone	T cell subsets	TrkA Δ MFI	NGF Δ MFI
GFP5	Th1	43	1.9
GFP8	Th1	26	1.0
GFP13	Th1	20	1.1
R1/28	Th1	27	1.7
R1/43	Th1	22	0.2
R1/44	Th1	21	0.1
GFT7	Th0	15	1.3
GFT16	Th0	27	1.7
GFT23	Th0	22	0.9
GFT27	Th0	24	0.8
GFT37	Th0	33	1.5
GTT25	Th2	21	1.2
GTT43	Th2	31	0.8
GTT47	Th2	35	1.6
DP25	Th2	25	1.5
GFT11	Th2	39	1.4

corresponding to residues 763 through 777 mapping within the cytoplasmic domain of human gp140trk sequence. This antibody, which reacts with both human and murine gp140trk, does not recognize TrkB and TrkC, the other members of the tyrosine kinase family. To detect nonspecific binding of antibodies, goat- or rabbit-purified immunoglobulins were used as control.

Flow cytometric analysis

Clonal T cell blasts were fixed in 4% phosphate-buffered paraformaldehyde for 10 minutes, after having been washed several times in buffered saline solution (BSS). The cells were then washed and permeabilized with BSS, 0.01 mol/L Hepes buffer solution, 0.1% saponin, and incubated for 45 minutes at 4°C with antibodies to NGF (10 μ g/ml) or TrkA (10 μ g/ml) diluted in BSS-saponin. T cell blasts, incubated with purified goat or rabbit IgG, were used as controls to evaluate the aspecific binding. After several washings in BSS-saponin, cells were exposed for 30 minutes at 4°C to fluorescein-conjugated mouse anti-goat (Cappel Laboratories, Cochranville, Pa.) or anti-rabbit (Boehringer Mannheim, Mannheim, Germany) IgG antibody. NGF- or TrkA-positive cells were quantified by an EPICS 541 flow cytometer (Coulter Electronics, Hialeah, Fla.). Fluorescence was excited by the 488 nm line of an argon-ion laser Innova 90 (Coherent, Palo Alto, Calif.) with a power of 50 mW and measured with a band-pass filter at 515 nm. Both logarithmic and linear signals were acquired from 5×10^3 cells per sample. Mean fluorescence intensity (MFI) of linear distribution was calculated, the specific signal for each sample was subtracted to the specific one, and results were expressed as increments relative to the controls (Δ MFI = (specific MFI – aspecific MFI)/aspecific MFI).

Quantification of NGF protein

To assess the amount of NGF secreted by ongoing and PHA-activated T cell clones, 10^6 /ml cells were cultured for 48 hours in medium conditioned by IL-2 in either the absence or presence of PHA (10 ng/ml). NGF levels were measured in culture supernatants by use of a highly sensitive two-site immunoenzymatic assay that recognizes human and murine

TABLE II. NGF levels (pg/ml) in culture supernatants of T cell clones subsets

Clone	T cell subsets	Ongoing	Activated
GFP5	Th1	12.8	13.0
GFP8	Th1	15.3	22.4
GFP13	Th1	5.3	5.0
R1/28	Th1	9.9	9.0
R1/43	Th1	5.0	2.0
R1/44	Th1	6.2	6.3
GFT7	Th0	n.d.	15.9
GFT16	Th0	n.d.	12.2
GFT23	Th0	n.d.	16.9
GFT27	Th0	n.d.	15.2
GFT37	Th0	n.d.	15.0
GTT25	Th2	8.7	9.1
GTT43	Th2	5.1	16.4
GTT47	Th2	6.3	9.5
DP25	Th2	5	5.4
GFT11	Th2	5.6	21.3

NGF and can detect concentrations as low as 5 pg/ml.²⁷ Briefly, polystyrene 96-well immunoplates (NUNC, Roskilde, Denmark) were coated with affinity-purified polyclonal goat anti-NGF antibody. Parallel wells were coated with preimmune goat IgG to evaluate the nonspecific binding. After overnight incubation at room temperature and 2 hours' incubation with the coating buffer of 0.05 mol/L (carbonate buffer pH7.6 in 1% bovine serum albumin), plates were washed with Tris-HCl at pH 7.4 (50 mmol/L, NaCl 200 mmol/L, 0.5% gelatin, 0.1% Triton X-100). The supernatants and the NGF standard solutions were distributed into the wells and left at room temperature overnight. The plates were washed and incubated with 4 mU/well anti- β -NGF-galactosidase (Boehringer Mannheim) for 2 hours at 37°C and, after washing, 100 μ l of substrate solution consisting of 4 mg chlorophenol red (Boehringer Mannheim) per milliliter of substrate buffer were added to each well. After an incubation of 2 hours at 37°C, optical density was measured at 575 nm by use of an ELISA reader (Dynatech Laboratories, Chantilly, Va.) and the values of standards and samples were corrected by subtraction of the background value due to nonspecific binding. Data were expressed as picograms per milliliter and all the assays were performed in triplicate.

Statistical evaluation

Nonparametric statistical analysis (Mann-Whitney U test) was performed to compare TrkA/NGF immunoreactivity and the NGF levels in the supernatants of the different T_H clones. Data are presented as mean $\pm$ SEM, and a p value $\leq$ 0.05 was considered statistically significant.

RESULTS

Our data show that human CD4+ T cell clones produce and release NGF and express the high-affinity receptor for this neurotrophin.

Microscopic analysis showed that every unstimulated T cell clone tested expressed high-affinity NGF receptors, as indicated by the specific anti-TrkA antibody staining. The immunohistochemical pattern observed in a representative T_{H2} clone is shown in Fig. 1, A. Quan-

titative evaluation by flow cytometric analysis confirmed that fixed T cell blasts from all the unstimulated clones expressed a very strong TrkA immunoreactivity (ranging from 16 to 44 times the control). No significant differences were evident between T_{H1} (mean Δ MFI = 27, range 43 to 21), T_{H0} (mean Δ MFI = 24, range 15 to 33), and T_{H2} clones (mean Δ MFI = 30, range 21 to 39) for their TrkA expression (Table I). Flow cytometry analysis of TrkA expression by a representative T_{H2} clone is shown in Fig. 1, C.

Immunohistochemical analysis with anti-NGF antibody on fixed T cell blasts of unstimulated clones clearly showed an intracytoplasmic accumulation of NGF (Fig. 2, A). Evaluation by flow cytometric analysis confirmed that all the unstimulated T cell clones stored NGF in their cytoplasm, showing a specific NGF immunoreactivity (1.1 to 2.9 times the control). Although two T_{H1} clones (R1/43 and R1/44) presented a very weak immunoreactivity (MFIs of 0.2 and 0.1, respectively), no significant differences were observed between T_{H1} (mean Δ MFI = 1, range 0.1 to 1.9), T_{H0} (mean Δ MFI = 1.2, range 0.8 to 1.7), and T_{H2} clones (mean Δ MFI = 1.3, range 0.8 to 1.6) (Table I). Flow cytometric analysis of cytoplasmic NGF expression by a representative T_{H2} clone is shown in Fig. 2, C.

As shown in Table II, low amounts of NGF were secreted by unstimulated T_{H1} and T_{H2} clones, with a mean production of 9.1 ± 1.7 and 6.1 ± 0.7 pg/ml, respectively. Stimulation with PHA resulted in a consistent increase (101%) of NGF secretion by T_{H2} clones (12.3 ± 2.9 pg/ml; $p < 0.05$). However, no significant difference was observed between the levels of NGF secreted in culture supernatants by ongoing and stimulated (9.6 ± 3.0 pg/ml) T_{H1} clones (Fig. 3), even though a mean increase of 65 ± 12 U/ml INF- γ was detected in the same supernatants of stimulated T_{H1} clones (data not shown). Detectable amounts of NGF (15.0 ± 0.8 pg/ml) were found in supernatants of T_{H0} cell clones stimulated with PHA but not in T_{H0} ongoing cells (Table II).

DISCUSSION

Our data demonstrate that human CD4+ T cell clones express NGF and TrkA receptors.

It has been well established that T_H lymphocytes play a key role in immune responses because they interact with different immune cells and modulate inflammatory processes.³⁶ In both mice and humans, T_H cells represent a functionally heterogeneous population, including at least three different functional subsets termed T_{H1} , T_{H2} , and T_{H0} based on their profile of cytokine secretion.³¹ T_{H1} cells produce IL-2, INF- γ , and TNF- β ; promote macrophage activation and delayed-type hypersensitivity reactions; and produce complement-fixing and opsonizing antibodies. T_{H2} cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13; provide B cells with optimal help for antibody production; and promote mast cell growth and eosinophil differentiation and activation.³⁷ In the absence of polarizing conditions, T_H cells (T_{H0} cells)

secreting both the T_{H1} - and T_{H2} -type cytokines usually arise and mediate intermediate effects.³⁶ In agreement with a previous observation in murine T_H clones,²³ our study showed that unstimulated human T_H clones expressed high-affinity NGF receptors, irrespective of their cytokine secretion profile. This observation is also consistent with previous reports showing NGF receptors on human resting lymphocytes^{11, 25, 26} and, indirectly, with data indicating that NGF can induce proliferation of human peripheral blood lymphocytes¹¹ and stimulates the expression of IL-2 receptors on these cells.^{12, 13} Taken together, these findings suggest a functional role for NGF receptors on human T cells.

Our data also show that all the T cell clones tested expressed cytoplasmatic immunoreactive NGF and that T_{H1} and T_{H2} clones release this neurotrophin in an unstimulated condition. NGF is released at a concentration that is biologically active on human basophils as demonstrated by *in vitro* studies.⁹

Upon mitogen stimulation, a different degree of NGF release and accumulation in culture supernatants was observed between T_{H1} and T_{H2} or T_{H0} clones. These data are partially consistent with a previous observation in murine T_H clones in which antigen stimulation increased NGF messenger ribonucleic acid expression in T_{H2} cells but not in T_{H1} cells.²³ On the other hand, the apparent lack of an increase in NGF levels in culture supernatants of stimulated human T_{H1} clones may either reflect an intrinsic failure of this functional cell subset or simply reflect a higher rate of NGF consumption peculiar to T_{H1} cells. The coexpression of NGF and its receptor on the same cell population has been demonstrated for neuronal cells and indicates the presence of an NGF autocrine circuit to support neuronal survival.³⁸⁻⁴⁰ Even though unclear at present, it seems likely that a similar mechanism also occurs in human T_H cells, as suggested for murine mast cells³² and lymphocytes.²³

Recent studies have reported an increased NGF expression in tissues of patients with systemic sclerosis⁴¹ and in the synovial fluid of patients with chronic autoimmune arthritis.²⁸ High circulating NGF levels were measured in murine schistosomiasis⁴² and in patients with systemic lupus erythematosus⁴³ or with allergic conjunctivitis.²⁹ More recently, we have reported increased circulating NGF levels in allergic diseases and asthma.³⁰ Interestingly, an important pathogenic role has been attributed to T_H cells producing T_{H2} -type cytokines and/or mast cells in all of these conditions.⁴⁴⁻⁴⁶ Indeed, some evidence suggests that NGF may act on cells able to modulate the inflammatory reaction in these diseases. NGF is involved in the induction of mast cell and basophil degranulation,^{8, 9} suppression of leukotriene C4 formation by eosinophils,⁴⁷ stimulation of lymphocyte proliferation and differentiation,¹⁰⁻¹³ and B cell immunoglobulin production.¹⁴ The possibility that NGF is preferentially involved in immune responses dominated by T_{H2} cytokines is a stimulating working hypothesis awaiting further investigations.

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