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Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma

(rhinoconjunctivitis/urticaria-angioedema/IgE/bronchial hyperreactivity/eosinophil cationic protein)

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ABSTRACT **Nerve growth factor (NGF) serum levels were measured in 49 patients with asthma and/or rhinoconjunctivitis and/or urticaria-angioedema. Clinical and biochemical parameters, such as bronchial reactivity, total and specific serum IgE levels, and circulating eosinophil cationic protein levels, were evaluated in relation to NGF values in asthma patients. NGF was significantly increased in the 42 allergic (skin-test- or radioallergosorbent-test-positive) subjects (49.7 ± 28.8 pg/ml) versus the 18 matched controls (3.8 ± 1.7 pg/ml; $P < 0.001$). NGF levels in allergic patients with asthma, rhinoconjunctivitis, and urticaria-angioedema were 132.1 ± 90.8, 17.6 ± 6.1, and 7.6 ± 1.8 pg/ml ($P < 0.001$, $P < 0.002$, and $P < 0.05$ versus controls), respectively. Patients with more than one allergic disease had higher NGF serum values than those with a single disease. When asthma patients were considered as a group, NGF serum values (87.6 ± 59.8 pg/ml) were still significantly higher than those of control groups ($P < 0.001$), but allergic asthma patients had elevated NGF serum levels compared with nonallergic asthma patients (132.1 ± 90.8 versus 4.9 ± 2.9 pg/ml; $P < 0.001$). NGF serum levels correlate to total IgE serum values ($\rho = 0.43$; $P < 0.02$). The highest NGF values were found in patients with severe allergic asthma, a high degree of bronchial hyperreactivity, and high total IgE and eosinophil cationic protein serum levels. This study represents the first observation (that we know of) that NGF is increased in human allergic inflammatory diseases and asthma.**

Nerve Growth Factor (NGF) is a well-characterized neurotrophic factor essential for survival, development, and maintenance of peripheral sympathetic and embryonic sensory neurons and of basal forebrain cholinergic neurons in the central nervous system (1, 2). Biological actions of NGF are mediated by low and high-affinity cell surface receptors (3, 4) that, upon binding and internalization of the ligand, trigger a cascade of biochemical and morphological events in NGF-target cells (2, 4). NGF displays a high degree of genetic, structural, immunological, and biological homology among the various species studies, including humans (5, 6). In addition to the neurotrophic activity, NGF has been reported to exert broader biological activities on nonneuronal cells. NGF is involved in the maturation and response of the integrated network of adaptive systems to offending stimuli and seems to exert a crucial effect on cells of the immune system (7, 8). For example, both low- and high-affinity NGF receptors are expressed on a variety of immune and inflammatory cells (8, 9). Thus, NGF enhances T- and B-cell-mediated immune responses (10), induces lymphocyte differentiation and mast cell (MC) proliferation, activates immune functions, and causes the release of soluble biological mediators from MCs (11–15).

NGF is produced by several cells including *in vitro* and *in vivo* MCs (1, 7, 8). Controversy exists, however, about the mechanisms regulating NGF synthesis and release, both in experimental animals and in humans. It has recently been reported that NGF accumulates in the dermis of systemic sclerosis patients (16) and that the level of NGF is increased in the synovial fluid of patients with chronic autoimmune arthritis (17) and in the cerebrospinal fluid of patients with multiple sclerosis (18). Studies indicate that the level of NGF in the bloodstream increases in patients affected by these diseases (8). In 1993, Bracci-Laudiero and coworkers demonstrated that patients affected with an active form of systemic lupus erythematosus show higher NGF serum levels than both patients with an inactive form and healthy control patients (19). We have recently reported that both plasma and inflamed tissues of patients affected by vernal keratoconjunctivitis also contain elevated levels of NGF and that this increase is associated with the number and distribution of MCs (20).

These findings, along with the observation that activated MCs represent an important source of NGF, prompted us to investigate whether the level of circulating NGF also increases in patients affected by other allergic diseases, such as asthma, rhinitis, and urticaria-angioedema, and eventually to correlate these changes with the clinical and pathophysiological features of these diseases.

METHODS

Patients. Forty-nine patients with asthma and/or rhinoconjunctivitis and/or urticaria-angioedema (18 males and 31 females; mean age of 33.7 years; age range of 5–72 years) and 18 age-matched controls were studied.

Allergy Diagnosis. Forty-two patients had clinical evidence of sensitization to common inhalant (e.g., grass, *Parietaria officinalis*, *Olea europea*, and *Dermatophagoides pteronyssinus*) or food (e.g., ovalbumin and lattoglobulin) allergens. Diagnosis of sensitization was made on the basis of at least one definitely positive skin prick test (wheal greater than that of the histamine response after subtraction of the diluent skin reaction area) associated with the presence of circulating IgE antibody (Unicap; Pharmacia) to the same allergen(s). On the basis of the standard clinical diagnostic criteria and of predominant clinical symptoms, 13 of the 42 allergic patients were

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Abbreviations: ECP, eosinophil cationic protein; MC, mast cell; NGF, nerve growth factor; PC20, provoking concentration of histamine causing a 20% decrease in forced expiratory volume per sec; RAST, radioallergosorbent test.

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diagnosed as having asthma (isolated in 4 cases, associated rhinitis in 4 cases, and with rhinoconjunctivitis in 5 cases), 15 were diagnosed as having rhinitis (associated with conjunctivitis in 8 cases and with both conjunctivitis and episodic asthma in 1 case), and 14 were diagnosed as having urticaria-angioedema (associated with episodic asthma in 2 cases and with rhinitis in 1 case). Allergic patients, selected for not having received any hyposensitization treatment, were studied out of the pollen season. Seven patients with asthma but negative skin tests and radioallergosorbent tests (RASTs) to common allergens were also studied. None of the patients had been receiving any kind of treatment at the time of blood collection; steroid treatment, if any, had been discontinued for at least 2 weeks before our study began. Eighteen age-matched, healthy subjects showing skin-test- and RAST-negative results to a panel of the eight more common inhalant and food allergens were used as controls.

Asthma Grading. The 20 asthmatic patients were classified on the basis of the Aas Score (21), which distinguishes five classes of bronchial asthma with respect to the clinical severity of the disease, from mild asthma with apparently normal lung functions (grade I) to chronic, incapacitating disease with continuous oral medications (grade V). On the basis of the Aas Score, five asthmatic patients fell within grade I, two fell within grade II, eight fell within grade III, and five fell within grade IV.

Bronchial Reactivity. Bronchial reactivity to histamine was measured in all asthmatic patients. Increasing doses of histamine (from 0.03 to 8 mg/ml in PBS, pH 7.4) were administered by a Wright nebulizer (Pari Provotest, Starnberg, Germany) for 2 min. Bronchial reactivity to histamine was expressed as the provoking concentration of histamine on the dose-response curve causing a 20% decrease of basal forced expiratory volume per sec (PC20). According to the value of PC20, asthmatic patients were classified as having severe (0.03–0.25 mg/ml), moderate (0.25–2.25 mg/ml), or mild (2.25–8 mg/ml) bronchial hyperreactivity, or normal bronchial reactivity (>8 mg/ml).

Serum IgE and Eosinophil Cationic Protein (ECP) Determination. Serum measurement of total IgE was performed in asthmatics by the paper radioimmunosorbent test (Pharmacia). ECP serum levels were also measured in asthmatic patients by a competitive radioimmunoassay (ECP-RIA, Kabi Pharmacia, Uppsala). ECP present in serum competes with a fixed amount of ^{125}I-labeled ECP for an anti-ECP antibody. The addition of a second antibody immunosorbent divides bound and free ECP; after centrifugation and decanting, the radioactivity in the pellet was measured by a gamma counter, as it is inversely related to the quality of ECP present in the sample.

NGF Serum Determination. NGF serum levels were measured in both asthma patients and controls by a highly sensitive, two-site, immunoenzymatic assay for both human and murine NGF capable of detecting concentrations as low as 5 pg/ml (22). Briefly, polystyrene 96-well immunoplates (Nunc) were coated with affinity-purified polyclonal goat anti-NGF antibody. Parallel wells were coated with preimmune goat IgG for evaluation of the monospecific signal. After overnight incubation at room temperature and 2 hr of incubation with the coating buffer (0.05 M carbonate buffer, pH 9.5, in 2% BSA), plates were washed with 50 mM Tris·HCl (pH 7.4), 200 mM NaCl, 0.5% gelatin, and 0.1% Triton X-100. After extensive washing, the diluted sera and the NGF standard solutions were distributed into the wells and left at room temperature overnight. The plates were washed and incubated with 4 milliunits of anti-β NGF-β-galactosidase per well (Boehringer Mannheim) for 2 hr at 37°C, and after another washing, 100 μl of substrate solution (4 mg of chlorophenol red per ml of substrate buffer; Boehringer Mannheim) was added to each well. After an incubation of 2 hr at 37°C, the optical density was measured at 575 nm using an ELISA reader (Dynatech), and the values of standards and samples were corrected by subtracting the background value due to nonspecific binding. Data are presented as mean values ± SE. However, in view of the non-Gaussian distribution of the individual values, nonparametric tests were used for statistical analysis. All measurements were performed in triplicate.

Analysis of variance for various diseases and allergens responsible for sensitizations was performed by the Kruskall–Wallis test. Statistical comparison of NGF values between groups was made by the Mann–Withney test. Correlation between variables was performed by the Spearman's correlation test. A P value of <0.05 was considered significant.

RESULTS

NGF serum values were significantly increased in allergic subjects (49.7 ± 28.8 pg/ml; $n = 42$) versus healthy controls (3.8 ± 1.7 pg/ml; $n = 18$; $P < 0.001$) (Fig. 1). Values measured in allergic patients with asthma, rhinoconjunctivitis, and urticaria-angioedema were, respectively, 132.1 ± 90.8, 17.6 ± 6.1, and 7.6 ± 1.8 pg/ml ($P < 0.001$, $P < 0.002$, and $P < 0.05$ versus controls) (Fig. 2). Interestingly enough, although NGF serum levels did not differ significantly in patients with different allergic diseases, patients with more than one allergic disease had higher NGF serum values than those with a single disease (Fig. 3).

No difference could be detected through variance analysis depending on the type of disease or allergen responsible for sensitization.

When asthma subjects ($n = 20$) were considered as a group (Table 1), NGF serum values were still significantly higher than those of controls (87.6 ± 59.8 pg/ml versus 3.8 ± 1.6 pg/ml; $P < 0.001$). Allergic (skin-test- or RAST-positive) asthmatic patients had higher NGF values than nonallergic (skin-test- or RAST-negative) asthmatic patients (132.1 ± 90.8 pg/ml versus 4.9 ± 2.9 pg/ml; $P < 0.005$) (Fig. 4); the latter did not differ significantly from controls (4.9 ± 2.9 pg/ml

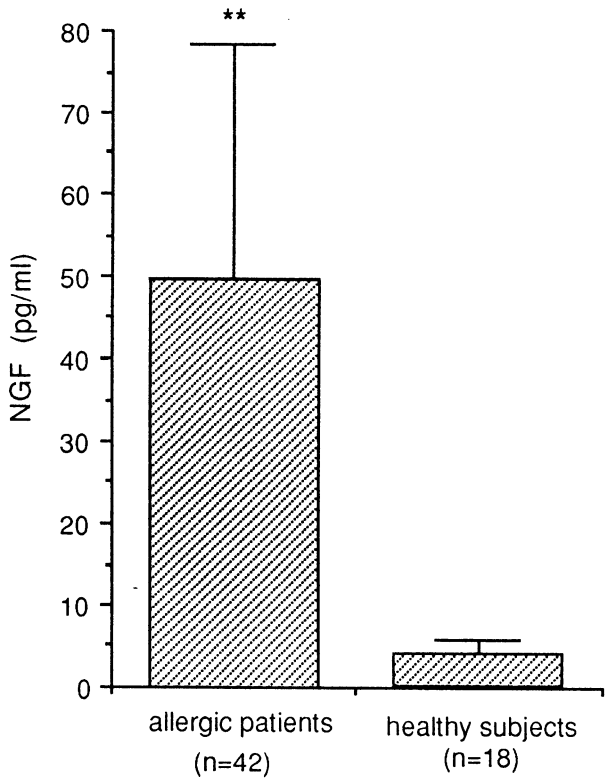

FIG. 1. NGF serum levels were significantly increased ($P < 0.001$) in allergic patients (49.7 ± 28.8 pg/ml) when compared with healthy subjects (3.8 ± 1.7 pg/ml).

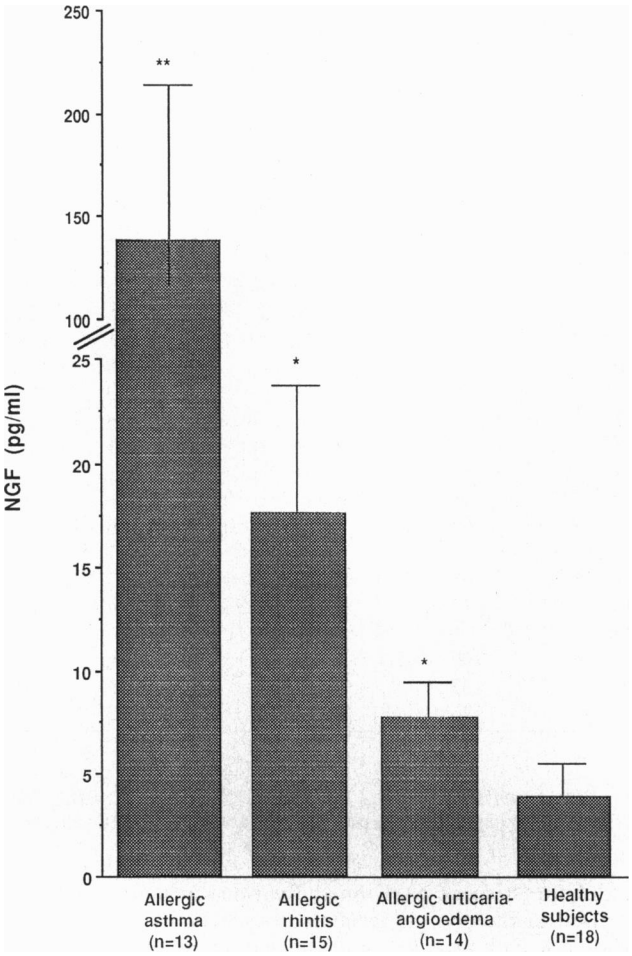

FIG. 2. NGF serum levels in allergic patients with asthma (132.1 ± 90.8 pg/ml), rhinoconjunctivitis (17.6 ± 6.1 pg/ml), and urticaria-angioedema (7.6 ± 1.8 pg/ml) were significantly increased when compared with healthy subjects ($P < 0.001$, $P < 0.002$, and $P < 0.05$, respectively).

versus 3.8 ± 1.6 pg/ml). Serum NGF values were significantly related to serum IgE values ($P < 0.02$), though with a low degree of correlation ($\rho = 0.43$). No significant correlation was observed between NGF serum values and PC20, or ECP in asthma patients.

Patients with a more severe asthma (Aas score III and IV) had higher mean NGF serum values than patients with mild forms (Aas score I and II; 112.0 ± 92.1 pg/ml versus 43.0 ± 18.4 pg/ml). Accordingly, asthmatic patients with hyperreactivity to histamine at the time of study had higher NGF serum values than normoreactive asthmatics (121.1 ± 99.7 pg/ml versus 37.3 ± 17.0 pg/ml). However, also in view of the high variability of values in relation to the number of subjects studied, these differences do not reach statistical significance. Interestingly, individual patients with severe allergic asthma, high degrees of bronchial reactivity at the time of study, high total serum IgE levels, and high serum ECP levels also had high NGF serum values (Table 1).

DISCUSSION

The presence of NGF in the bloodstream of experimental animals has long been a matter of controversy, in spite of the extensive evidence regarding the structure and function of this molecule. The first clear documentation came only after studies involving strong stressing stimuli in adult mice (23).

In humans, despite the suggested relevant role of NGF in the immune-endocrine-nervous network (8, 9, 24), as far as

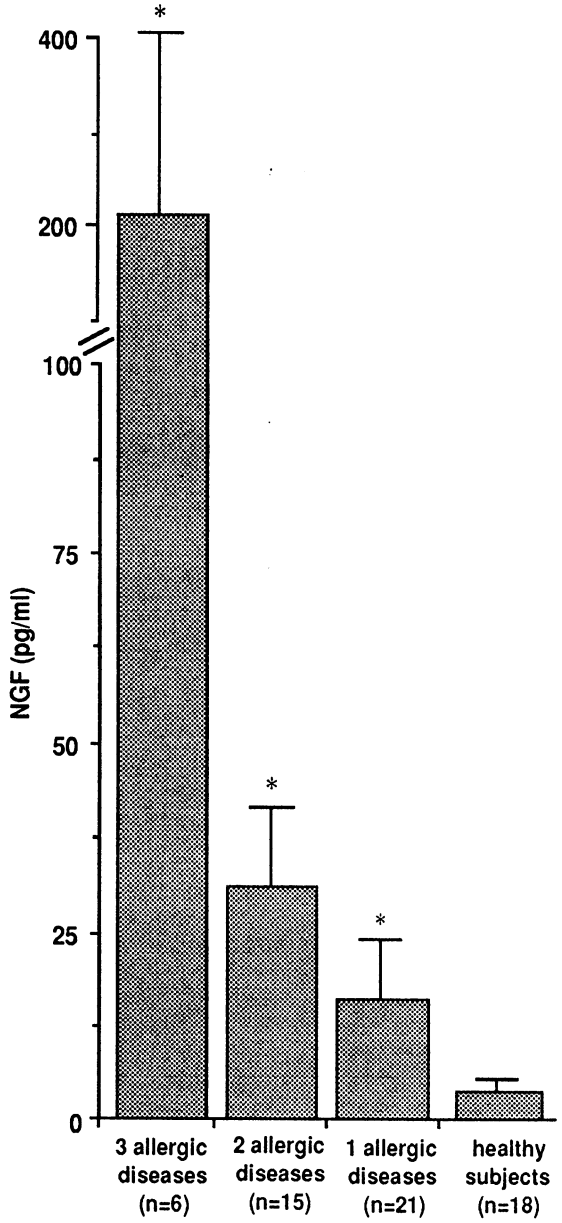

FIG. 3. NGF serum levels in patients with one or more allergic diseases. Although NGF serum levels were significantly increased in any group of patients studied versus controls, the highest NGF values were observed in patients with 3 associated allergic diseases (217.65 ± 199.25 pg/ml).

we know increased levels of NGF in the circulation have been reported only in systemic lupus erythematosus (19) and in 15 cases of vernal kerato conjunctivitis (20), a rare form of allergic conjunctivitis in which NGF serum mean values are 10-fold higher than in normals and 2-fold higher than in systemic lupus erythematosus. This study indicates that the increase of serum NGF is not confined to vernal kerato conjunctivitis but also extends to other major allergic diseases, thus representing the first evidence (in diseases with high prevalence and social relevance) of the possible significance of measuring NGF serum levels. Interestingly enough, if we consider that NGF accumulates in the tissues of systemic sclerosis patients and increases in the synovia of patients with chronic autoimmune arthritis, all the data available seem to indicate that NGF is increased in conditions with a Th2 profile and/or involvement of MCs and other inflammatory cells.

Table 1. Characteristics of patients with asthma

		Anamnesis		Clinical features		Serological data			
Patient no., sex	Age, years	Associated allergic disease	Duration of disease, years	Bronchial reactivity*	Asthma grading†	Total IgE, milliunits/l	RAST‡	ECP, μg/l	NGF, pg/ml
1, M	21	—	10	Severe	IV	695	Grass, Dpt.	34.9	0
2, F	26	—	15	Mild	III	316	Dpt.	31.6	21.9
3, F	41	—	30	Moderate	III	136	Grass	17.6	99.3
4, M	43	—	7	Normal	I	74	Grass, *Olea*	9.5	6.8
5, F	15	Rhinitis	10	Mild	III	395	Grass	33.5	20.8
6, F	25	Rhinitis	8	Normal	I	>1000	Grass, Par. off., Dpt.	24.1	118.5
7, F	44	Rhinitis	16	Normal	II	195	Egg	31.8	102.9
8, F	46	Rhinitis	20	Moderate	IV	89	Grass	8.2	61.1
9, F	18	Rhinoconjunctivitis	5	Mild	I	158	Par. off., Grass	32.8	2.9
10, F	22	Rhinoconjunctivitis	10	Normal	I	266	Grass, Dpt.	21.1	28.0
11, F	26	Rhinoconjunctivitis	16	Moderate	III	>1000	Grass, Dpt.	72.1	1213.4
12, M	41	Rhinoconjunctivitis	14	Normal	II	161	Grass, Par. off.	13.8	0
13, M	60	Rhinoconjunctivitis	34	Normal	I	54	Grass, Par. off.	22.6	42
14, M	31	—	10	Severe	IV	45	Neg.	33.7	0
15, M	34	—	3	Severe	III	108	Neg.	14.7	0
16, F	37	—	10	Severe	IV	37	Neg.	48.4	20.5
17, F	56	—	15	Mild	III	5	Neg.	11.6	0
18, F	44	—	8	Moderate	III	100	Neg.	21.6	6.9
19, M	39	—	5	Mild	II	167	Neg.	62.4	0
20, M	72	—	30	Severe	IV	126	Neg.	14.3	6.8

*According to PC20 histamine.
†According to the Aas Score.
‡Serum measurements of specific IgE include rye grass (Grass), *Dermatophagoides pteronyssinus* (Dpt.), *Parietaria officinalis* (Par. off.), *Olea europaea* (*Olea*), milk, and egg proteins. We considered RAST-positive patients who showed even a class 1 positivity for at least one of the allergens tested. Neg., Negative.

In our study, the common variable that seems to be more significantly associated with high NGF serum values appears to be the occurrence of an IgE-mediated MC degranulating process. In fact, skin-test-positive patients have significantly increased serum NGF values independently from the disease or the type of allergen responsible for sensitization. Therefore, the MC, Th2 type, eosinophilic allergic inflammation appears to go along with high NGF serum values.

The association between allergic diseases and increased serum NGF values might suggest two different interpretation hypotheses. The first hypothesis refers to a possible modulatory role of NGF in allergic inflammation. In fact, NGF has been shown to promote MC colony growth and differentiation in both humans and experimental animals (12, 25, 26). It increases the number of MCs in rat tissues (27) and induces mediator release from these cells (15). T cells are also stimulated by NGF in rats (28), and activated CD4 T-cell clones possess high-affinity NGF receptors (29). Similarly, B cells proliferate and differentiate upon NGF stimulation (30), with preferential IgG4 production (31), an isotype which undergoes a Th2 type of regulatory mechanism (interleukin 4, interleukin 13) which is similar to that of IgE (32, 33). Eosinophil is a key cell in allergic inflammation (34). NGF promotes eosinophil differentiation from peripheral progenitors and suppresses LTC4 synthesis in humans (12, 35). NGF also promotes colony growth and differentiation of basophils in both mouse and human (12, 26). It synergizes granulocyte macrophage-colony stimulating factor, interleukin 3, and interleukin 5 in increasing histamine content and differentiation of basophil and MC lines (26, 36). Moreover, NGF primes IgE-mediated mediator release from human circulating basophils (14) and promotes neutrophil chemotaxis and superoxide production both *in vitro* (37) and *in vivo* in mouse (38). Finally, NGF promotes differentiation of monocytes from peripheral blood progenitors (12) and extends its action to fibroblasts, as shown by an accelerated wound healing after administration in mouse (39, 40). All these effects of NGF are consistent with a potential modulatory role of NGF on the various inflammatory cells involved in the allergic inflammatory network and with the hypothetic pathogenetic significance of increased NGF serum values in allergic diseases.

As an alternative interpretation hypothesis, we might suggest that high NGF serum levels in allergic diseases are a secondary event, representing a product of inflammatory cells involved in these pathologic conditions. In fact, MCs that have a primary role in allergic diseases and close structural and functional relationships with nerves (41) are able to synthesize, store, and release NGF (7). This hypothesis, however, does not exclude the possibility of a modulatory role of NGF in allergic inflammation, through an effect of NGF on other inflammatory cells or by an autocrine-positive feed-back mechanism induced by MC-derived NGF (9).

Independently from their pathogenetic significance, increased NGF serum levels could well have an additional potential clinical significance in allergic disease states. In fact, increasing attention is at present directed to markers of MC activation to monitor MC degranulation and severity of allergic inflammation at the clinical level (42). In this area of research, specific MC products, such as tryptase, although present in secretion (43), are not detectable in significant levels in blood, except for in the massive degranulation process (44). On the other hand, nonspecific MC products, such as histamine, have been shown to cause methodological problems in detection and limited clinical significance. The high variability of NGF values shown in allergic patients in this study should prompt further investigations to determine whether NGF might represent a clinical marker of MC degranulation and allergic inflammation.

In our study, NGF serum levels were particularly high in asthma patients. Extensive evidence concerning the inflammatory nature of this disease has been accumulated, and MCs, eosinophils, and T lymphocytes play a primary role in this evidence (45). Recent *in vitro* studies showing that NGF increases nerve conductance and sensitivity (46) suggest that

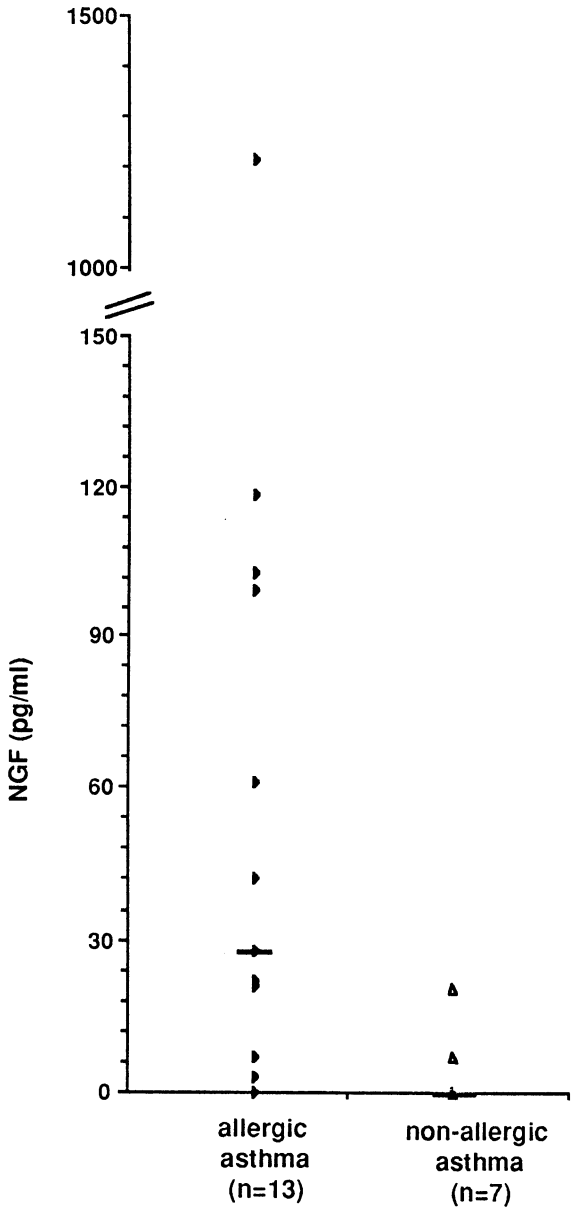

FIG. 4. NGF serum levels in allergic (skin-test- or RAST-positive) asthmatic patients were significantly higher than levels in nonallergic (skin-test- or RAST-negative) asthmatic patients (mean = 132.1 ± 90.8 pg/ml, median = 28.0 pg/ml versus mean = 4.9 ± 2.9 pg/ml, median = 0 pg/ml; $P < 0.005$).

NGF might be involved in the "nerve hyperalgesia" associated with reflex symptoms of asthmatic patients (47). We did not find a significant correlation between NGF serum levels and bronchial hyperreactivity or ECP serum levels, a possible marker of allergic inflammation in asthma (48), in the population sample studied. However, the higher NGF serum values observed in severe and hyperreactive allergic asthmatics, although they did not reach statistical significance in this study, should encourage further investigations with *ad hoc* designed protocols on the clinical significance of high NGF levels in a polyfactorial and heterogeneous disease.

In conclusion, the immune, endocrine, and nervous systems represent a single integrated body defense system in which any imbalance of one of the three components possibly reflects on secondary or regulatory effects of the other two. The finding that NGF serum levels are increased in allergy and asthma patients introduces a new element, suggesting that this neurocytochine might play a role in the complex and still largely unexplored inflammatory network involved in allergy and asthma.

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