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Original article

Effect of anti-nerve growth factor on early and late airway responses in allergic rats

Background: The increased production of nerve growth factor (NGF) has been associated with allergen-induced airway hyperresponsiveness and enhanced airway inflammation in experimental models of asthma. The aim of this study was to investigate whether a local application of anti-NGF to the lungs may affect the allergen-specific early (EAR) and late (LAR) airway responses to ovalbumin (Ova) of Ova-sensitized brown Norway rats.
Methods: Rats were sensitized systemically with Ova and were boosted twice intratracheally with Ova aerosol using a microsprayer. Two hours before every boost, the animals were pretreated either with aerosolized anti-NGF or with a control antibody. On day 21, all animals were challenged with inhalational Ova aerosol and pulmonary resistance was recorded in anesthetized, orotracheally intubated animals during the early and late asthmatic responses. In addition, differential cell counts from bronchoalveolar lavage and serum immunoglobulin E (IgE) levels were determined 48 h post-Ova challenge.
Results: Pretreatment with anti-NGF significantly attenuated the EAR but had no significant effect on the LAR. Serum IgE levels and inflammatory cell influx into the lungs were not affected by anti-NGF pretreatment.
Conclusion: The data from this study suggest that NGF is directly involved in the development of the EAR without affecting the inflammatory airway response or LAR.

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Neurotrophins are a family of structurally related proteins that regulate neuronal function and have been associated with tissue inflammation and hyperalgesia. There is growing evidence from human and animal studies that neurotrophins such as nerve growth factor (NGF) may also be involved in the development of allergic disorders including asthma (reviewed in 1–3). Although the exact role of NGF is not yet understood, several studies postulate that NGF is included in the neuroimmune regulation of airway inflammation and airway hyperreaction (AHR) (1–3). The enhanced NGF levels found during allergic inflammation are believed to be produced by numerous immune cells including mast cells, macrophages, T cells and B cells (reviewed in 1, 2) as well as other resident lung cells including fibroblasts and epithelial cells (4, 5). These cells also interact with NGF via the high-affinity receptor TrkA or the low affinity receptor p75. In that context, NGF acts as a cytokine-like factor by increasing interleukin-4 (IL-4) and IL-5 production, activating eosinophils and augmenting mast cell function (1–3). These effects are considered to contribute to asthma pathophysiology including alterations of airway reactivity. By enhancing neuropeptide release from sensory neurons, NGF could furthermore contribute to an altered airway function because of neurogenic inflammation (6). First evidence of an involvement of NGF in early airway responses (EAR) came from a study demonstrating that anti-NGF inhibits EAR in guinea-pigs (7). In the present study, we assessed the response of the airways in ovalbumin (Ova)-sensitized and boosted rats by pretreatment with anti-NGF to directly investigate the role of NGF in the early and late effector phases of asthma.

Material and methods

Animals

Pathogen-free inbred male Brown Norway (BN) rats (Charles River, Sulzfeld, Germany), 7–8 week of age and weighing 150–170 g, were used in all experiments. All experimental animal studies conformed to the guidelines of the National Research Council (NRC) Guide.

Sensitization protocol

On day 0, BN rats were sensitized with a s.c. injection of 0.1 mg Ova (grade V, Sigma, Taufkirchen, Germany) precipitated with 20 mg of aluminum hydroxide gel in 0.25 ml normal saline. An adjuvant consisting of 6×10^9 heat-killed *Bordetella pertussis* organisms was injected i.p. at the same time. On days 7 and 14, the anesthetized animals (halothane 4% for approximately 2 min; Eurim-Pharm, Piding, Germany) were boosted intratracheally with 30 μg of aerosolized Ova. Two hours before every boost, the anesthetized animals ($n = 7$–8 each group, anesthesia as above)

were pretreated with 200 μg of aerosolized anti-NGF (rabbit anti-NGF 2.5 S, Sigma) or with rabbit IgG (Sigma) dissolved in phosphate buffer saline (PBS) in a volume of 0.1 ml. Aerosols were generated with a microsprayer (type IA-1C; PennCentury, Philadelphia, PA) that was inserted into the orotracheal tube.

Determination of respiratory function

Pulmonary resistance (RL) was assessed in anesthetized and orotracheally intubated rats as previously described (8). On day 21, allergen-specific early (EAR) and late (LAR) airway responses to Ova aerosol (inhaled dose: 60 μg over 13 min) were determined. Aerosols (MMAD: 1.7 μm) were generated by a computer-controlled aerosol generator system (Bronchy III, Fraunhofer ITEM, Germany) (8).

Determination of total IgE in serum

Plates were coated with mouse monoclonal antibody to rat IgE (MCA 193, Serotec, Düsseldorf, Germany), blocked and incubated with sera and standard (rat IgE, PRP07, Serotec). Peroxidase-labelled anti-IgE antibody (MCA 187P, Serotec) was added, and the plates were developed after addition of the peroxidase substrate O- phenylenediamine dihydrochloride OPD (Sigma). The reaction was stopped by 1 N HCL (Merck, Darmstadt, Germany) and the plates were read with an enzyme-linked immunosorbent assay (ELISA) plate reader at 490 nm (8).

Assessment of leukocyte distribution, nerve growth factor and histology

Assessment of leukocyte distribution in bronchoalveolar lavage (BAL) fluid was performed 48 h after allergen challenge with 5×5 ml of ice cold saline. Total cell counts were determined with a Casy cell counter (Schaerfe System, Reutlingen, Germany). Differential cell counts were assessed on May–Grünwald–Giemsa stained slides based on standard morphologic criteria. After BAL, the lungs were carefully removed and were inflated and fixed with 4% (w/v) formaldehyde via the trachea and stored in 4% formaldehyde. Lung tissues were paraffin embedded, and sections were stained with hematoxylin and eosin (H & E). Immunohistological staining for anti-NGF antibodies were performed using paraffin embedded lung samples of each animal that were cut and sections (4 μm) were deparaffinized. An alkaline phosphatase anti-alkaline phosphatase (APAAP) standard technique was used to detect rabbit immunoglobulins (anti-NGF) (Dako). NGF was measured in serum and BAL using a commercially available ELISA kit (Roche, Mannheim, Germany). The NGF detection limit was 5 pg/ml.

Statistics

The groups were compared with the unpaired Student's *t*-test. Values for all measurements were given as mean ± SD. *P*-values <0.05 were considered significant.

Results

Effect of anti-NGF on early and late airway responses to Ova challenge

The measurement of respiratory function during EAR demonstrated a significant lower increase in RL to Ova challenge in anti-NGF pretreated animals compared with control animals ($P < 0.05$) (see Fig. 1). Figure 2 displays characteristic time-response courses illustrating the changes in lung resistance (RL), tidal volume (VT) and frequency (*f*) during the EAR in rats with and without pretreatment with anti-NGF. LAR peaked at 5–6 h after Ova challenge. All animals that mounted an EAR also demonstrated an LAR, as indicated by significantly enhanced RL values over baseline values. The degree of the LAR was not significantly different from that of the EAR in both groups. However, there was no significant difference in RL values during the LAR between anti-NGF treated animals and controls indicating that pretreatment with anti-NGF antibody had no significant effect at distance of the Ova challenge.

Effect of anti-NGF on airway inflammation

The allergen-sensitized and challenged animals showed comparable serum levels of total IgE and had similar numbers of eosinophils, lymphocytes, macrophages and neutrophils in BAL fluid (Table 1). There were no detectable NGF titers in serum 48-h post-Ova challenge. No significant difference in levels of NGF in BAL fluid 48 h after Ova challenge were found between groups (63 ± 21 anti-NGF *vs.* 71 ± 23 pg/ml control). In addition, lung histology showed a similar degree of

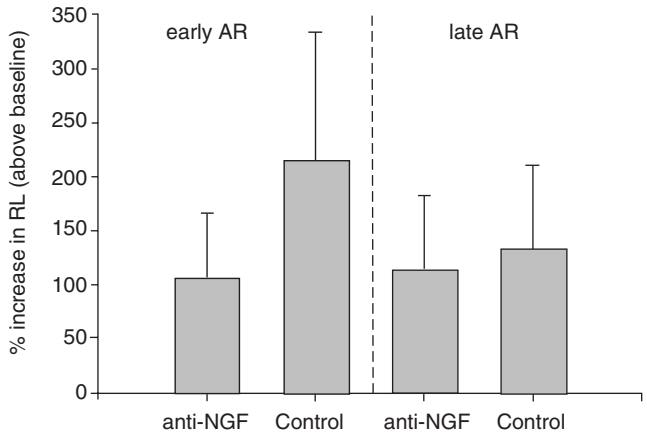

Figure 1. Assessment of EAR and LAR to Ova challenge ($c = 135$ mg/m^3 for 13 min) in allergic rats with and without anti-NGF pretreatment. Pretreatment with anti-NGF significantly attenuated the increase in RL values during the EAR when compared with control rats (*$P < 0.05$). In contrast, no significant differences were observed during the LAR between both groups. AR is expressed as percentage change in RL to baseline values which were taken as 0% (baseline values: anti-NGF pretreatment: RL: 0.23 ± 0.06 cm H_2O·s/ml;. control rats: RL: 0.23 ± 0.05 cm H_2O·s/ml). Values are means ± SD from eight animals per group.

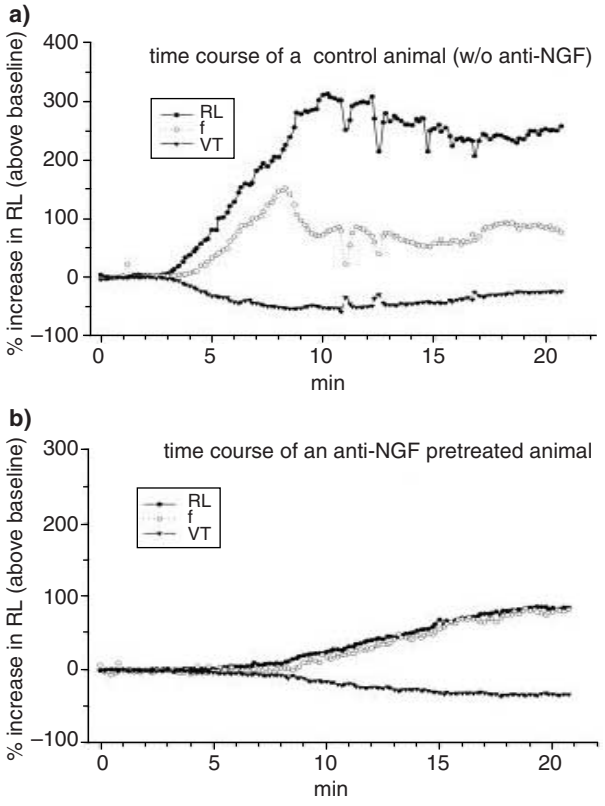

Figure 2. Representative time-course of an EAR illustrating the increases in RL and *f* values with a concomitant decline in VT during and following Ova aerosol challenge of an allergic rat with (a) and without (b) anti-NGF-pretreatment. AR is expressed as percentage changes in *f*, VT and RL to baseline values, which were taken as 0%. Baseline values were (a): VT: 1.2 ml; RL: 0.16 cm H_2O·s/ml; *f*: 76 breaths per minute. (b): VT: 1.0 ml; RL: 0.29 cm H_2O·s/ml; *f*: 72 breaths per minute.

perivascular and peribronchial inflammation predominantly consisting of eosinophils and lymphocytes in all investigated animals of both groups (Fig. 3).

The immunohistological staining of the aerosolized anti-NGF rabbit antibodies in the lung 48-h post-Ova challenge revealed complete clearance of the antibodies at this time point (data not shown).

Discussion

This study showed that inactivation of NGF by a specific antibody alone is sufficient to significantly attenuate the magnitude of acute bronchoconstriction in a BN rat model of allergic asthma. In contrast, there was no significant effect of anti-NGF neither on the late asthmatic response nor on airway inflammation. The latter finding coincides with a previous study in Ova-sensitized BALB/c mice reporting no effect of anti-NGF treatment on the cellular inflammatory response in BAL fluid (9). However, a recent report in allergic mice showed an anti-inflammatory effect of anti-NGF treatment on the inflammatory parameters in BAL fluid (10). In that respect it is noteworthy that under T helper-2 (Th-2) conditions, NGF seems to act as a proinflammatory Th-2 cytokine, whereas anti-inflammatory effects of NGF have been described for an experimental Th-1 model of autoimmune encephalomyelitis (11). Although the exact mechanisms by which NGF and its receptors may affect airway function are incompletely understood, inhibition of EAR by anti-NGF might be explained by several mechanisms. First, interactions between NGF and mast cell activation via the TrkA or p75 receptor may cause EAR by inducing enhanced mediator release from mast cells (1–3). In addition, mast cells are located in close proximity to sensory neurons. Suggested interactions between nerve and mast cells involve neurotrophins such as NGF that may serve as signaling molecules in the transduction of neurogenic inflammation by elevating sensory neuropeptide production in the airways (1–3). Neuropeptides like substance P operate through the high-affinity neurokinin (NK)-1 receptor and are

Table 1. Individual inflammatory status of the animals

Parameter	IgE (μg/ml)		Eosinophils (×10^6)		Lymphocytes (×10^6)		Neutrophils (×10^6)		Macrophages (×10^6)	
Treatment group	Control	Anti-NGF	Control	Anti-NGF	Control	Anti-NGF	Control	Anti-NGF	Control	Anti-NGF
1	1.50	3.54	2.14	4.95	0.22	0.46	0.19	0.32	6.92	8.27
2	3.95	4.42	4.06	3.42	0.34	0.27	0.79	0.14	9.66	9.68
3	1.78	4.74	5.47	4.99	0.00	0.00	0.53	0.23	10.07	9.03
4	2.31	4.48	2.94	3.41	0.03	0.00	0.15	0.35	8.05	9.53
5	3.95	3.59	4.35	4.20	0.00	0.13	0.29	0.34	8.13	8.45
6	3.11	2.69	7.10	4.16	0.19	0.00	0.47	0.32	6.53	7.88
7	6.91	4.40	2.68	3.27	0.00	0.10	0.20	0.39	9.31	6.05
8	3.19		2.80		0.06	0.46		0.32		8.27
Mean	3.34	3.98	3.94	4.06	0.11	0.18	0.37	0.30	8.38	8.40
SD	1.60	0.68	1.57	0.67	0.12	0.18	0.22	0.07	1.26	1.07

IgE levels were determined in serum. Cell numbers were counted in BAL fluid and cell differentiation was performed on May–Grünwald–Giemsa stained cytospin preparations.

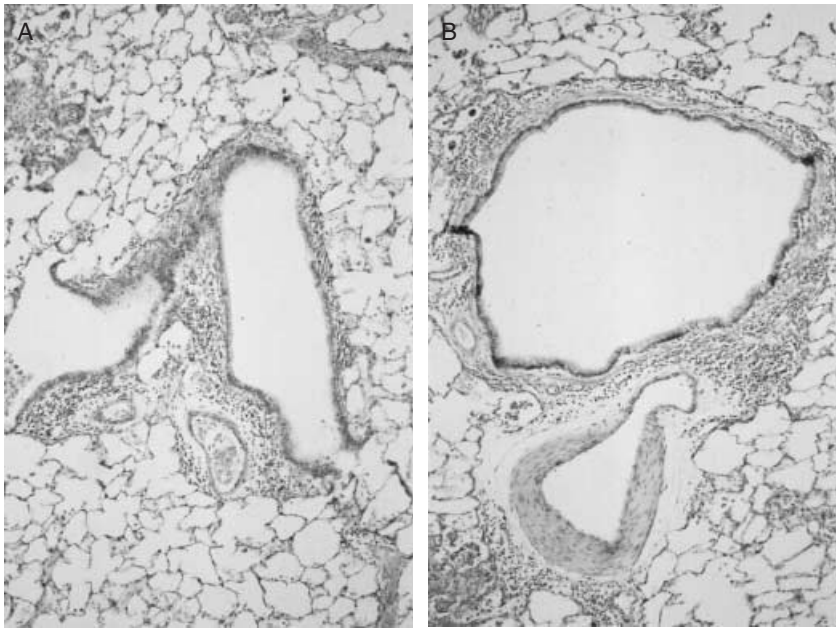

Figure 3. Representative H & E stained lung sections from Ova-challenged animals without treatment (A) and after anti-NGF treatment (B). Perivascular and peribronchial leukocyte infiltration consisting of eosinophils, neutrophils and mononuclear cells was present in both groups.

capable of stimulating mast cell activation and degranulation, which finally leads to EAR (1–3, 12). Antagonism of NGF might affect these cells, modulate IgE-mediated responses, cause the inhibition of the release of stimulating factors and indirectly block mast cell degranulation. Secondly, NGF-mediated stimulation of sensory afferent nerve endings in the airway epithelium may trigger both a reflex increase in efferent cholinergic activity and an antidromic release of neuropeptides via a short-circuited local loop reflex (2, 3). Other possible mechanisms include stimulation of afferent neuropeptide-containing nerve endings that are exposed because of inflammation or enhanced sensitivity of airway smooth muscle to the bronchoconstricting neuropeptides (2, 3). The administration of anti-NGF, however, did not completely inhibit EAR. In contrast to the inhibitory effect of aerosolized anti-NGF on EAR, pretreatment with aerosolized anti-NGF neither had a significant effect on pulmonary resistance during LAR nor on airway inflammation in BAL fluid or lung histology. A number of studies described enhanced NGF levels in BAL fluid up to 1–2 days after segmental allergen challenge in humans (13, 14). The reasons why we could not demonstrate a decrease in NGF-levels in BAL fluid by anti-NGF treatment 48-h post-Ova challenge in rats are unclear and remains to be elucidated further. One explanation might be that anti-NGF antibody temporarily blocks the bioavailibility of NGF but does not abolish enhanced NGF levels produced upon allergen stimulation. Moreover, it is still unclear, whether the presence of NGF in BAL fluid *per se* infer their causal role in the pathogenesis of the late phase response. Our data obtained with allergic rats are supported by several animal studies reporting that treatment with i.v. NGF increased histamine-induced acute bronchoconstriction in guinea-pigs (12) and that pretreatment with anti-NGF diminished the EAR both in allergen-sensitized guinea-pigs and in mice (8, 10). Together, these findings may suggest that NGF rather contributes to the development of EAR than to that of LAR. In summary, this study provides *in vivo* evidence that antagonism of the NGF pathway effectively modifies early phase bronchoconstriction without affecting the LAR or airway inflammation in allergic BN rats. We conclude that, among multiple other immune-regulating factors, NGF is directly involved in the development of the EAR in a rat model of allergic asthma.

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