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Atropine-enhanced, antigen challenge-induced airway hyperreactivity in guinea pigs is mediated by eosinophils and nerve growth factor

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Verbout NG, Jacoby DB, Gleich GJ, Fryer AD. Atropine-enhanced, antigen challenge-induced airway hyperreactivity in guinea pigs is mediated by eosinophils and nerve growth factor. *Am J Physiol Lung Cell Mol Physiol* 297: L228–L237, 2009. First published May 15, 2009; doi:10.1152/ajplung.90540.2008.—Although anticholinergic therapy inhibits bronchoconstriction in asthmatic patients and antigen-challenged animals, administration of atropine 1 h before antigen challenge significantly potentiates airway hyperreactivity and eosinophil activation measured 24 h later. This potentiation in airway hyperreactivity is related to increased eosinophil activation and is mediated at the level of the airway nerves. Since eosinophils produce nerve growth factor (NGF), which is known to play a role in antigen-induced airway hyperreactivity, we tested whether NGF mediates atropine-enhanced, antigen challenge-induced hyperreactivity. Antibody to NGF (Ab NGF) was administered to sensitized guinea pigs with and without atropine pretreatment (1 mg/kg iv) 1 h before challenge. At 24 h after challenge, animals were anesthetized, vagotomized, paralyzed, and ventilated. Electrical stimulation of both vagus nerves caused bronchoconstriction that was increased in challenged animals. Atropine pretreatment potentiated antigen challenge-induced hyperreactivity. Ab NGF did not affect eosinophils or inflammatory cells in any group, nor did it prevent hyperreactivity in challenged animals that were not pretreated with atropine. However, Ab NGF did prevent atropine-enhanced, antigen challenge-induced hyperreactivity and eosinophil activation (assessed by immunohistochemistry). This effect was specific to NGF, since animals given control IgG remained hyperreactive. These data suggest that anticholinergic therapy amplifies eosinophil interactions with airway nerves via NGF. Therefore, therapeutic strategies that target both eosinophil activation and NGF-mediated inflammatory processes in allergic asthma are likely to be beneficial.

anticholinergic; asthma; muscarinic receptors; parasympathetic nerves; neurotrophins

IN ASTHMA, ACETYLCHOLINE RELEASE from parasympathetic nerves that control airway smooth muscle contraction is increased, leading to airway hyperreactivity (6). The same is true in animal models of asthma (10, 21, 22, 34). Acetylcholine release is inhibited by neuronal M₂ muscarinic receptors present on parasympathetic nerves, and loss of M₂ receptor function is characteristic of asthma in humans (1, 35) and of airway hyperreactivity in animals (18, 21). Blockade of M₃ receptors on airway smooth muscle should therefore be beneficial in asthma. However, it has been suggested that anticholinergic therapy may potentiate inflammatory mechanisms in airway disease (23, 48). We have shown that muscarinic blockade by atropine before and during antigen challenge

actually exacerbates airway hyperreactivity measured in guinea pigs 24 h later. This increased airway hyperreactivity is associated with increased eosinophil activation in the airways (48).

Eosinophilic inflammation of the lungs is a prominent feature of asthma and is associated with airway hyperreactivity in experimental animals. Recent reports of the efficacy of eosinophil depletion by means of a monoclonal antibody to IL-5 (Ab IL-5) in asthmatic patients with sputum eosinophilia support the role of eosinophils in asthma pathogenesis (27, 36). Antigen challenge increases eosinophils in the lungs and around airway nerves of guinea pigs (5, 14, 48). After antigen inhalation, activated eosinophils degranulate near airway nerves, releasing major basic protein (MBP), an endogenous and selective antagonist for neuronal M₂ receptors (29), resulting in increased acetylcholine release and airway hyperreactivity (18, 21). Inhibition of eosinophil localization to airway nerves or neutralization of MBP protects against airway hyperreactivity (12–14, 17, 20).

Atropine-enhanced, antigen challenge-induced hyperreactivity is vagally mediated, but it is not associated with M₂ receptor dysfunction (48). However, atropine-enhanced, antigen challenge-induced hyperreactivity is dependent on the presence of eosinophils, since eosinophil depletion with Ab IL-5 prevents hyperreactivity and increased eosinophil activation in the airways (48). Therefore, atropine-enhanced, antigen challenge-induced hyperreactivity is mediated by interactions of airway nerves with eosinophil-derived factors released during degranulation and cytolysis.

Allergic inflammation is associated with increased neurotrophins in the lungs and bronchoalveolar lavage of humans and experimental animals (2, 7, 47, 49). In particular, nerve growth factor (NGF) is an inflammatory mediator that plays a role in allergic airway disease (16). There are multiple sources of NGF in the lung, including bronchial epithelium, pulmonary fibroblasts, bronchial smooth muscle, and several inflammatory cells, including eosinophils (45). NGF expression is increased in eosinophils isolated from allergic patients (39, 40), and NGF concentration is positively correlated with allergic disease severity, airway hyperreactivity, and eosinophil mediator release (38). NGF is known to mediate airway hyperreactivity by increasing substance P expression in sensory nerves (8, 47). NGF has also been demonstrated to induce substance P expression in airway parasympathetic nerves in guinea pigs (28) and ferrets (52). Since eosinophils are a source of NGF and are located in close proximity to airway nerves, we tested whether NGF plays a role in eosinophil-mediated atropine-enhanced, antigen challenge-induced hyperreactivity.

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METHODS

Animals. Specific pathogen-free female Dunkin-Hartley guinea pigs (150–200 g body wt; Elm Hill Breeding Labs, Chelmsford, MA) were shipped in filtered crates and kept in high-efficiency particulate-filtered air. Guinea pigs were fed a normal diet and handled in accordance with the standards established by the US Animal Welfare Acts set forth in National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committees at Oregon Health and Science University.

Sensitization and challenge with antigen. Guinea pigs were sensitized to ovalbumin (10 mg/kg ip; Sigma-Aldrich) every other day for a total of three injections, as previously described (20). At 3 wk after the last ovalbumin injection, guinea pigs were pretreated with the antihistamine pyrilamine (0.5 mg/kg ip; Sigma-Aldrich) 1 h before challenge and exposed to aerosolized ovalbumin (2.5%) for 5 min or until signs of respiratory distress appeared, in which case antigen challenge was immediately halted. After antigen challenge, all guinea pigs received isoproterenol (1 mg/kg ip; Sigma-Aldrich).

Treatments. Guinea pigs were treated with atropine (1 mg/kg ip; Sigma-Aldrich), a nonselective muscarinic antagonist, or saline 1 h before antigen challenge and again 6 h after antigen challenge (18 h before physiological measurements). In some animals, a goat IgG antibody specific to recombinant human β -NGF (10 μ g/kg ip; R & D Systems) or negative control normal goat IgG (10 μ g/kg ip; R & D Systems) was administered 1 h before antigen challenge.

Measurements of pulmonary inflation pressure. Experiments were conducted 1 day after antigen challenge, by which time the effects of atropine had subsided (48). Guinea pigs were anesthetized with urethane (Sigma-Aldrich) at 1.9 g/kg ip; at this dose, urethane produces a deep anesthesia of 8- to 10-h duration (24), although none of these experiments lasted longer than 4 h.

Physiological studies were performed as previously described (20). Jugular veins were cannulated bilaterally for administration of drugs, and one internal carotid artery was cannulated for measurement of blood pressure and heart rate. The trachea was cannulated, and animals were mechanically ventilated at a respiratory rate of 100 breaths/min, with a tidal volume of 2.5 ml. Animals were paralyzed by continuous succinylcholine infusion (10 μ g $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ iv; Sigma-Aldrich). Pulmonary inflation pressure was measured via a sidearm of the tracheal cannula. Bronchoconstriction was measured as the increase in pulmonary inflation pressure above the basal inflation pressure produced by the ventilator.

Measurement of postjunctional muscarinic receptor function on airway smooth muscle. Responsiveness of airway smooth muscle muscarinic receptors was assessed by administration of increasing doses of acetylcholine (1–10 μ g/kg iv; Sigma-Aldrich) and measurement of bronchoconstriction. The vagus nerves were cut to eliminate acetylcholine-induced reflex responses (50).

Vagal reactivity. Anesthetized, ventilated, and paralyzed guinea pigs were vagotomized, and distal portions of vagi were stimulated electrically via platinum electrodes. Electrical stimulation of the vagus nerves (1–25 Hz, 10 V, 0.2-ms pulse duration) for 5 s at 45-s intervals produced frequency-dependent bronchoconstriction and bradycardia that recovered on cessation of electrical stimulation. Administration of atropine (1 mg/kg iv) at the end of each experiment confirmed that vagally induced bronchoconstriction was mediated by cholinergic nerves.

Neuronal M_2 receptor function. Animals were pretreated with guanethidine (2 mg/kg iv; Sigma-Aldrich) to deplete norepinephrine. After 30 min, vagi were stimulated electrically (15 Hz, 0.2-ms pulse duration) for 3 s at 40-s intervals, with voltage adjusted to elicit reproducible bronchoconstrictions of 8–16 mmH $_2$ O before administration of the muscarinic (M_2 receptor-selective) antagonist gallamine (Sigma-Aldrich), and the effects on vagally induced bronchoconstriction were measured. Gallamine antagonizes M_2 receptors; thus cumulative doses of gallamine (0.1–10 mg/kg iv) potentiate vagally induced

bronchoconstriction in a dose-dependent manner. The degree to which gallamine increases vagally mediated bronchoconstriction is a measure of neuronal M_2 receptor function.

Bronchoalveolar lavage and peripheral blood analysis. After physiological measurements were made, the lungs were lavaged with five aliquots of 10 ml of warm PBS containing 100 μ M isoproterenol (Sigma-Aldrich) in situ via the tracheal cannula. Recovered lavage fluid was centrifuged, cells were resuspended in PBS, and total cells were counted using a hemocytometer. Aliquots of the cell suspension were centrifuged onto glass slides and stained for differential analysis. Whole blood was taken from the carotid artery via a heparinized syringe and placed in 0.1 N hydrochloric acid to lyse red blood cells. Total peripheral blood leukocytes were counted with a hemocytometer, and cell differentials were obtained from a blood smear.

Histological evaluation of eosinophils. At the end of the experiments, animals were given heparin (2,500 U iv; American Pharmaceutical Partners) and exsanguinated by perfusion of the jugular vein with warm saline while blood was drained from the cannulated carotid artery. Once the lungs were white, they were removed and inflated with zinc-buffered formalin (Anatech) and fixed overnight at 4°C. Transverse sections from two lobes of the lungs were embedded in paraffin for histology.

Nerves in the lungs were detected immunohistochemically using a mouse monoclonal antibody against protein gene product (PGP) 9.5 (Biogenesis), as previously described (5). Tissue sections were dewaxed, treated with antigen unmasking solution (Vector Laboratories), and blocked in 10% normal goat serum for 1 h at 37°C. Slides were incubated at 4°C for 24 h with primary antibody against PGP 9.5 (1:1,000 dilution in 10% normal goat serum in PBS). Tissue sections were quenched with 3% H $_2$ O $_2$ in methanol, blocked with a casein-based blocking solution (CAS-Block, Zymed), and incubated for 90 min at 37°C with the secondary antibody biotinylated goat anti-mouse IgG (Vector Laboratories; 1:400 dilution in 5% normal goat serum). Slides were washed with PBS, incubated with an avidin-linked horseradish peroxidase substrate (ABC Elite, Vector Laboratories), and washed again with PBS. PGP 9.5 staining was visualized by incubation of tissues with the chromagen diaminobenzidine and nickel (Vector Laboratories), which stained airway nerves black. Eosinophils were visualized by counterstaining for 45 min with a 1% solution of Chromotrope 2R (Sigma-Aldrich), which stained eosinophils red. Slides were washed in tap water, dehydrated in graded ethanol solutions, cleared in xylene, and permanently mounted with Cytoseal-60 (Richard-Allan Scientific).

The number of eosinophils within the walls of five different cartilaginous airways per animal (3–5 animals per treatment group) was counted. Airways were photographed with a digital camera attached to a Nikon microscope, and airway area was measured using Metamorph imaging software (version 6.2, Universal Imaging). Total area of smooth muscle within airway walls and below the lamina propria was measured, and the total number of eosinophils within that area was counted in consecutive high-power fields. Eosinophils within 8 μ m of an airway nerve (approximately the diameter of 1 eosinophil) were also counted. Thus the number of eosinophils per square millimeter was calculated for each treatment group, and the proportion of eosinophils associated or not associated with airway nerves was determined.

Total MBP deposition in lungs. Eosinophil MBP in lung sections was detected using a rabbit polyclonal antibody against guinea pig MBP (Ab MBP) (32). Lungs were fixed, embedded, sectioned, dewaxed, treated with antigen unmasking solution, and blocked as described above. Slides were incubated at 4°C for 24 h with Ab MBP (1:1,000 dilution in goat serum), washed with PBS, and then incubated for 90 min at 37°C with goat anti-rabbit IgG (Molecular Probes) labeled with Alexa Fluor 555. Slides were washed, mounted under aqueous medium with 4,6-diamidino-2-phenylindole (Vector Laboratories), and stored at 4°C in the dark. Negative control slides were treated as described above without primary antibody.

Table 1. Effect of pretreatment with atropine, Ab NGF, or atropine + Ab NGF on resting heart rate, systolic blood pressure, and pulmonary inflation pressure 24 h after antigen challenge

Group	n	Heart Rate, beats/min	Blood Pressure, mmHg		Pulmonary Inflation Pressure, mmH ₂ O
			Systolic	Diastolic	
<i>Without Ab NGF</i>					
Control	6	299±5	39±3	21±2	128±9
Challenge	5	295±8	46±2	25±2	136±10
Challenge + atropine	5	293±9	44±3	19±1	140±10
<i>With Ab NGF</i>					
Control	5	284±5	38±2	22±3	129±14
Challenge	4	291±9	43±2	24±4	153±33
Challenge + atropine	5	293±4	45±3	22±1	135±5

Values are the means ± SE; n, number of guinea pigs. Ab NGF, antibody to nerve growth factor.

Slides were coded, and all remaining steps were carried out under blinded conditions. Airways were photographed under identical conditions, and images were analyzed for MBP deposition using Meta-morph imaging software. The airway lamina propria, smooth muscle, and any outer connective tissue attached or contiguous with the smooth muscle were included in the analysis. Airway epithelium, cartilage, blood vessels, and alveoli were excluded.

MBP deposition was quantified as previously described (48). Background fluorescence intensity was measured in a noncellular region of each airway lumen, and values were subtracted from each image. A threshold for MBP staining was measured using the negative control of the airway with the strongest MBP signal. A serial section containing this airway was stained without primary antibody, and average fluorescence was measured. This value set the lower limit of the calibration scale for all subsequent measurements. Only fluorescence above background and above MBP intensity threshold was measured. Total intensity was calculated by multiplying mean fluorescence intensity by the area containing positive MBP signal. Average fluorescence per square micrometer was determined by dividing total intensity by the total area of the airway. Values are means of three to five animals per group, with replicates of five airways per animal.

Data analysis and statistics. Values are means ± SE. In vivo, the whole frequency response to nerve stimulation and dose response to gallamine were compared using two-way ANOVA for repeated measures with Bonferroni's post hoc correction. Histological data for tissue eosinophils, peripheral leukocytes, lavage leukocytes, and MBP content in the airways were analyzed by one-way ANOVA with

Bonferroni's post hoc correction. Mortality data were analyzed for significance by χ^2 test. $P < 0.05$ was considered significant. Statistical data were analyzed with Kaleidagraph (version 4.01, Synergy Software) or Statview 4.5 (Abacus Concepts).

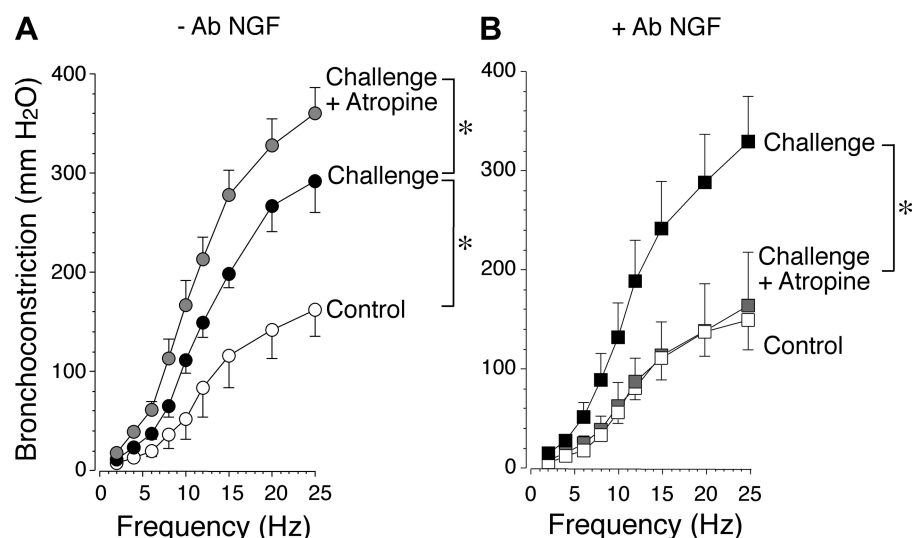
RESULTS

Treatments. Challenged animals were pretreated with the antihistamine pyrilamine (0.5 mg/kg ip) 1 h before antigen challenge, and isoproterenol (1 mg/kg ip) was administered immediately after challenge. All sensitized guinea pigs exhibited symptoms of acute respiratory distress with acute antigen challenge, and the incidence of mortality in saline-treated guinea pigs was not different from that in previous studies. In contrast, mortality following antigen challenge was significantly greater in animals treated with atropine (69.6%) or Ab NGF (63.6%) than in those treated with saline (28.6%) or atropine + Ab NGF (37.5%). Responses of nonsensitized control guinea pigs treated with pyrilamine or isoproterenol 24 h before challenge were not different from responses of untreated control animals.

Pulmonary baselines. At 24 h after antigen challenge, there was no difference among groups in resting pulmonary inflation pressure or any other parameter (Table 1).

Responsiveness of airway smooth muscle. Acetylcholine-induced bronchoconstriction was dose related, with a maxi-

Fig. 1. Atropine-enhanced, antigen challenge-induced hyperreactivity is reversed by pretreatment with nerve growth factor (NGF) antibody (Ab NGF). In nonsensitized nonchallenged control animals, electrical stimulation of both vagus nerves increased bronchoconstriction (measured as an increase in pulmonary inflation pressure) in a frequency-dependent manner (control, A) that was not changed by Ab NGF treatment 24 h before challenge (control, B). Antigen challenge of guinea pigs significantly increased vagally induced bronchoconstriction, which was further potentiated by atropine pretreatment (A). Atropine-enhanced, antigen challenge-induced hyperreactivity was prevented in animals treated with Ab NGF before challenge (challenge + atropine, B). Pretreatment with IgG did not prevent atropine-enhanced, antigen challenge-induced hyperreactivity (maximum bronchoconstriction at 25 Hz was 350 ± 30 mmH₂O, $n = 3$). Ab NGF alone did not prevent airway hyperreactivity in challenged animals (challenge, B). Values are means ± SE ($n = 4-6$). * $P < 0.05$, entire dose response vs. comparison dose response.



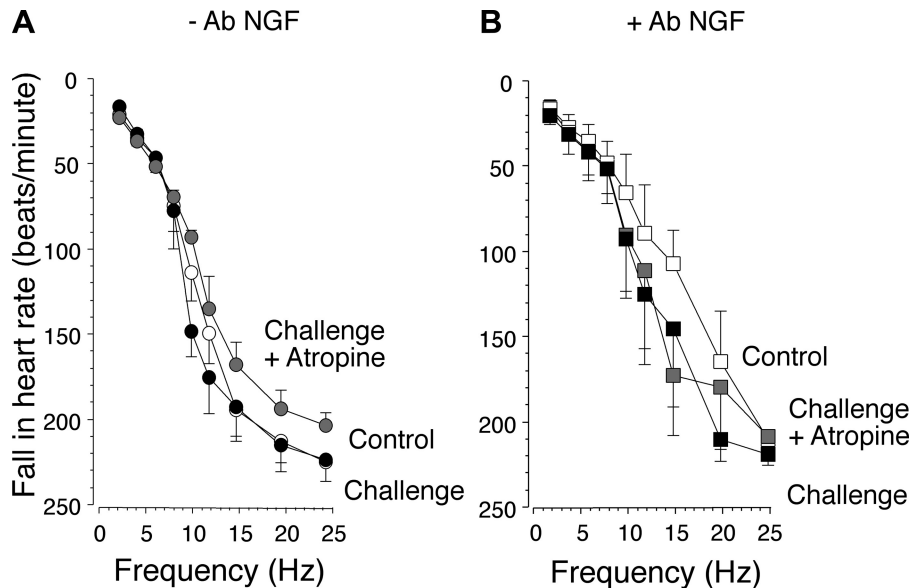


Fig. 2. A: electrical stimulation of both vagus nerves caused frequency-dependent bradycardia [control ($\circ$)] that was not changed by antigen challenge (challenge) or atropine pretreatment (challenge + atropine). B: vagally induced bradycardia was not significantly altered by pretreatment with Ab NGF in control, challenged, or challenged + atropine-treated guinea pigs. Values are means $\pm$ SE ($n = 4-6$).

maximum response of 212 ± 48 mmH₂O in control animals (10 μ g/kg iv, $n = 4$); this response was not altered by pretreatment with Ab NGF (maximum response of 243 ± 18 mmH₂O, $n = 5$). Thus Ab NGF does not change contractility of the airway smooth muscle.

Airway hyperreactivity. In nonsensitized nonchallenged control animals, electrical stimulation of both vagus nerves increased bronchoconstriction in a frequency-dependent manner (Fig. 1A) that was not changed by treatment with Ab NGF 24 h before challenge (Fig. 1B). Since atropine pretreatment does not alter vagally induced bronchoconstriction in control guinea pigs (48), this group was not included in this study. Antigen challenge of guinea pigs significantly increased vagally induced bronchoconstriction, which was further potentiated by atropine pretreatment (Fig. 1A).

Atropine-enhanced, antigen challenge-induced hyperreactivity was prevented in animals treated with Ab NGF before challenge (Fig. 1B). Pretreatment with control IgG did not prevent atropine-enhanced, antigen challenge-induced hyperreactivity (maximum bronchoconstriction at 25 Hz was 350 ± 30 mmH₂O, $n = 3$). In contrast, Ab NGF alone did not prevent airway hyperreactivity in challenged animals (Fig. 1B). Thus

atropine-enhanced, antigen challenge-induced hyperreactivity in antigen-challenged guinea pigs is mediated by NGF.

Bradycardia. Electrical stimulation of both vagus nerves caused frequency-dependent bradycardia that was not altered by antigen challenge or atropine pretreatment (Fig. 2A). Ab NGF did not affect bradycardia among any groups (Fig. 2B). Thus vagal hyperreactivity following antigen challenge is limited to the airways and does not extend to the heart.

M₂ receptor function. Gallamine, an M₂ receptor-selective antagonist, potentiated vagally induced bronchoconstriction in control guinea pigs in a dose-dependent manner, demonstrating M₂ receptors were functional (Fig. 3A). In antigen-challenged animals, vagally induced bronchoconstriction was not potentiated by gallamine, demonstrating that M₂ receptors were dysfunctional. In contrast, M₂ receptor dysfunction was partially protected by atropine pretreatment in challenged animals, suggesting that atropine-enhanced, antigen challenge-induced hyperreactivity is not mediated by neuronal M₂ receptor dysfunction. Pretreatment with Ab NGF did not significantly affect the ability of gallamine to potentiate vagally induced bronchoconstriction in challenged animals with or without atropine pretreatment. In contrast, the ability of gallamine to potentiate

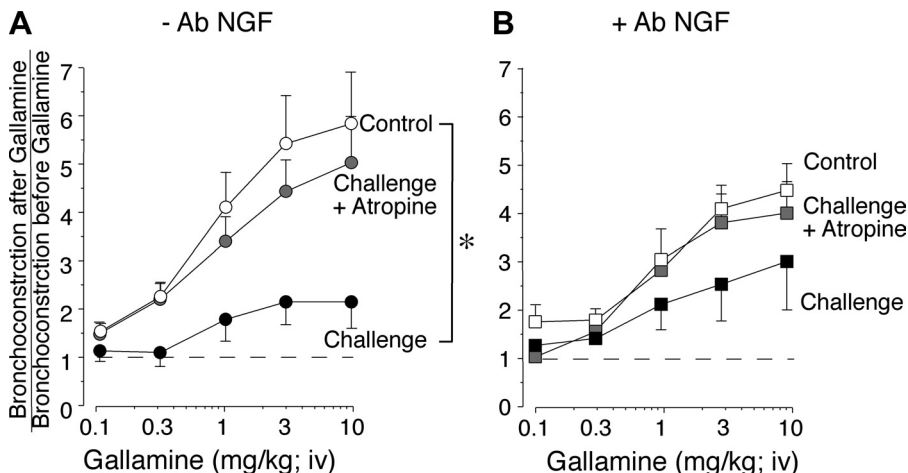
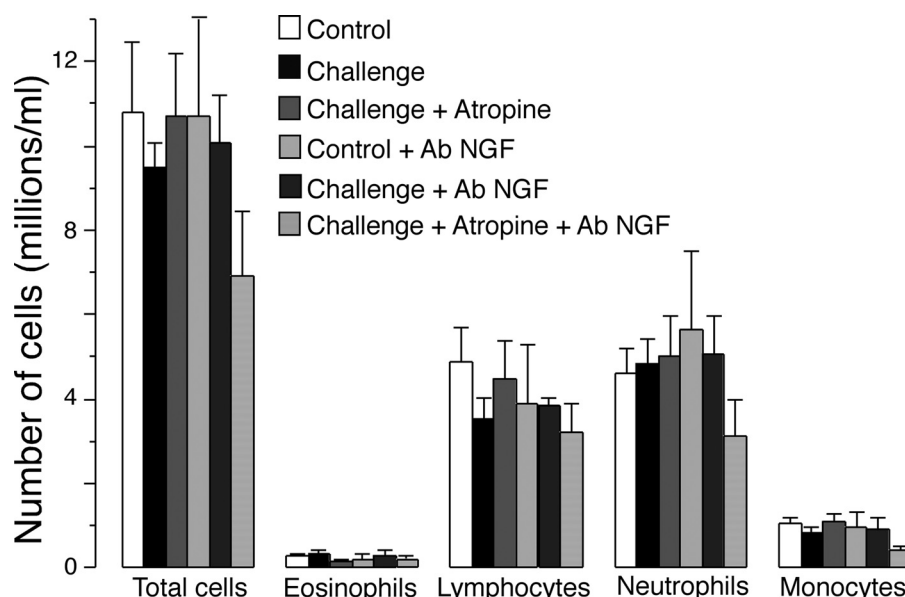


Fig. 3. A: gallamine, an M₂ muscarinic receptor-selective antagonist, potentiated vagally induced bronchoconstriction in control guinea pigs in a dose-dependent manner, demonstrating functional M₂ receptors (control). In antigen-challenged animals (challenge), vagally induced bronchoconstriction was not potentiated by gallamine, demonstrating that M₂ receptors were dysfunctional. M₂ receptor dysfunction was partially protected by atropine pretreatment in challenged animals (challenge + atropine), demonstrating that atropine-enhanced, antigen challenge-induced hyperreactivity is not mediated by neuronal M₂ receptor dysfunction. B: pretreatment with Ab NGF did not significantly affect the ability of gallamine to potentiate vagally induced bronchoconstriction in control, challenged, or challenged + atropine-treated guinea pigs. Values are means $\pm$ SE ($n = 4-6$). * $P < 0.05$, entire dose response vs. dose response in controls.

Fig. 4. There were no differences in circulating leukocyte populations among control, challenged, or challenged + atropine-pretreated guinea pigs with or without Ab NGF pretreatment. Values are means $\pm$ SE ($n = 4-6$).



vagally induced bronchoconstriction in control animals pretreated with Ab NGF was modestly decreased; however, this effect was not statistically significant (Fig. 3B).

Inflammation. There were no differences among leukocyte populations in blood (Fig. 4), nor were there were differences in bronchoalveolar lavage cells (Fig. 5), among control, challenged, or challenged + atropine-pretreated guinea pigs with or without Ab NGF pretreatment.

Histological staining revealed that eosinophils in airways and around nerves were significantly increased by antigen challenge (Fig. 6). Atropine pretreatment decreased total eosinophils in the airways (Fig. 6F) and around nerves (Fig. 6G) in antigen-challenged guinea pigs. Ab NGF did not significantly alter the antigen-induced increase in eosinophils or the atropine-mediated decrease in antigen-induced eosinophilia.

MBP deposition. Atropine pretreatment significantly increased eosinophil MBP deposition in airways of challenged guinea pigs. Ab NGF prevented this increase in MBP in atropine-pretreated animals (Fig. 7).

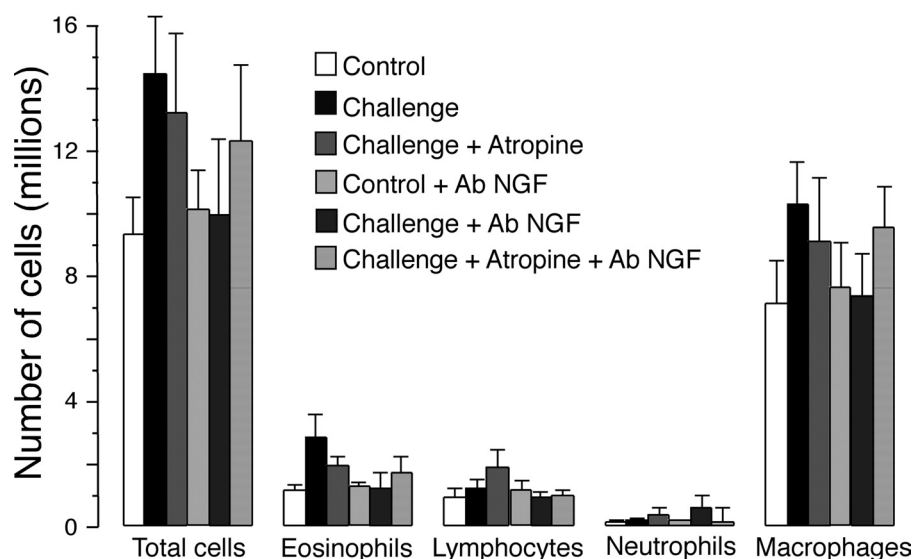
DISCUSSION

Antigen-induced airway hyperreactivity is significantly potentiated in animals treated with atropine 1 h before challenge (Fig. 1A), as we previously showed (48). Atropine-enhanced, antigen challenge-induced hyperreactivity is vagally mediated, since acetylcholine-induced bronchoconstriction is not potentiated by atropine pretreatment, also confirming previous findings (48).

Ab NGF did not affect airway tone or smooth muscle contractility (Table 1). However, blocking NGF before antigen challenge did prevent atropine enhancement of antigen challenge-induced hyperreactivity (Fig. 1). Ab NGF did not prevent hyperreactivity in antigen-challenged animals that were not treated with atropine. Thus atropine enhancement of antigen challenge-induced hyperreactivity is mediated by NGF.

Atropine pretreatment doubled challenge-induced mortality in antigen-challenged guinea pigs (from 28.6% to 69.6%), which coincided with potentiation in airway hyperreactivity

Fig. 5. There were no differences in bronchoalveolar lavage leukocyte populations among control, challenged, or challenged + atropine-pretreated guinea pigs with or without Ab NGF pretreatment. Values are means $\pm$ SE ($n = 4-6$).



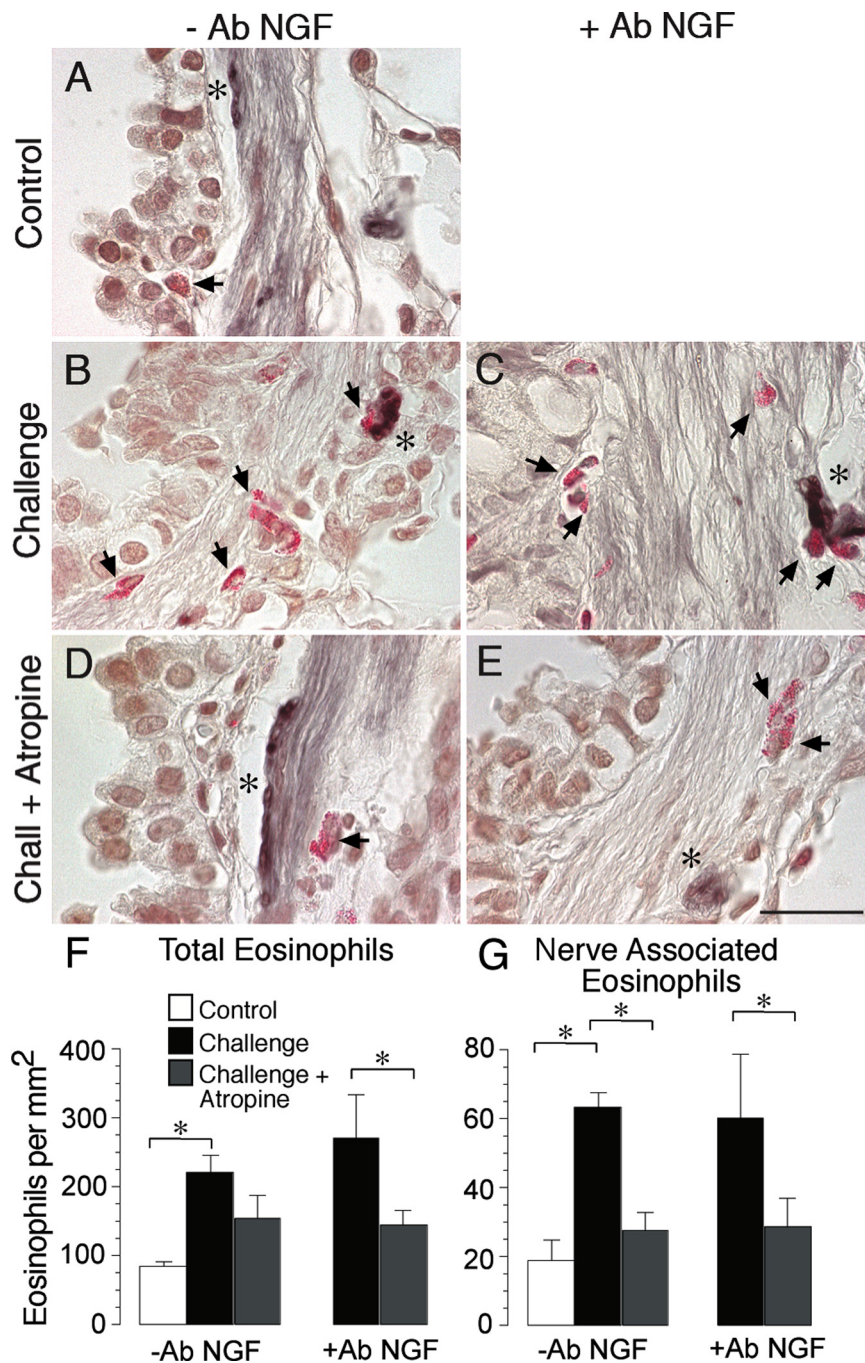


Fig. 6. Atropine pretreatment decreased eosinophils in airways of antigen-challenged animals. A–E: cross sections of guinea pig bronchi. Nerves were labeled with antibody to protein gene product (PGP) 9.5 (black, *), and eosinophils were counterstained with Chromotrope 2R (red, arrows). There were few eosinophils in lungs or around nerves in control guinea pigs (A). After antigen challenge, eosinophils were present within smooth muscle and in close proximity to nerves of non-Ab NGF-pretreated (B) and Ab NGF-pretreated (C) animals. Fewer eosinophils were observed within the lungs or in close proximity to nerves in animals treated with atropine before antigen challenge (D) and in animals treated with atropine + Ab NGF. Scale bar, 50 μ m. F and G: quantification of eosinophil accumulation in the airways. Antigen challenge (challenge) significantly increased eosinophils in lungs (F) and around nerves (G) compared with control. Pretreatment with atropine (challenge + atropine) decreased the antigen-induced increase in eosinophils in airways (F) and especially around nerves (G). Ab NGF did not significantly alter the antigen-induced increase in eosinophils or the atropine-mediated decrease in antigen-induced eosinophilia. Values (means $\pm$ SE, $n = 4-5$) are expressed as number of eosinophils/mm² airway smooth muscle (F) and number of eosinophils within 8 μ m of an airway nerve/mm² (G). * $P < 0.05$.

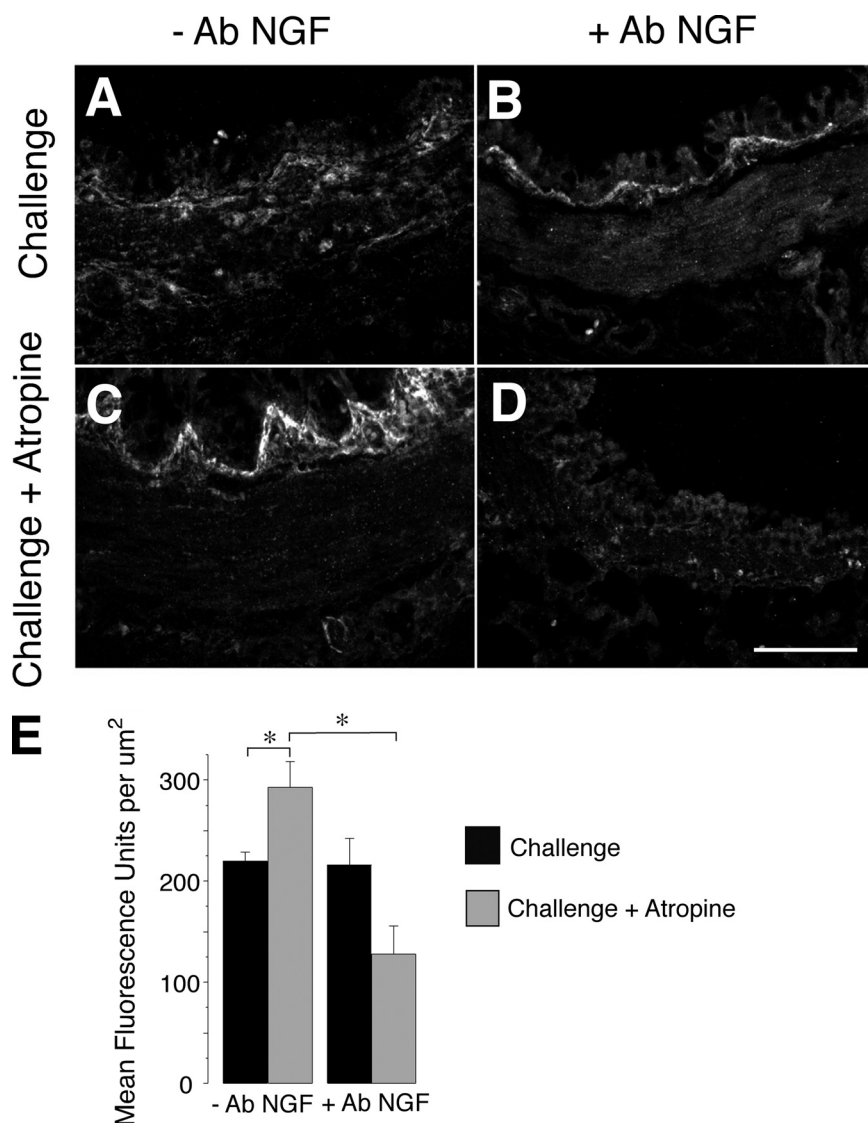
and increased eosinophil activation 24 h later. In animals treated with Ab NGF + atropine before challenge, mortality was reduced to 37.8%, which similarly coincided with protection from airway hyperreactivity and decreased eosinophil activation. Thus mortality risk appears to mirror physiological responses measured 24 h later.

Although antigen-induced airway hyperreactivity in guinea pigs is caused by eosinophil-induced loss of neuronal M₂ receptor function (12, 13, 18, 19, 21), atropine-enhanced, antigen challenge-induced hyperreactivity is not associated with neuronal M₂ receptor dysfunction, confirming our previous findings (48). In the present study, M₂ receptor function and dysfunction did not appear to be significantly affected by Ab NGF (Fig. 3). Even

though the gallamine responses in control animals compared with challenged animals were not statistically different in the presence of Ab NGF (Fig. 3B), the trend among these groups is very similar to gallamine responses in the absence of Ab NGF (Fig. 3A). Thus it is probable that additional experiments would yield statistical significance between these groups. However, given that the mechanism of atropine-enhanced potentiation is independent of neuronal M₂ receptor function, additional animals were not added to the study, since the conclusions drawn from these experiments would remain the same.

Antigen challenge increases eosinophil activation in the lung (13, 25, 48). As we previously described (48), muscarinic blockade by atropine at the time of antigen challenge further

Fig. 7. Ab NGF decreased eosinophil major basic protein (MBP) deposition in challenged animals pretreated with atropine. Eosinophil MBP deposition within airways of challenged guinea pigs was labeled using antibody to MBP, and fluorescence intensity was evaluated in gray scale. *A–D*: representative photographs of airways from challenged animals (*A*) that were pretreated with Ab NGF (*B*), atropine (*C*), or Ab NGF + atropine (*D*). Scale bar, 50 μ m. *E*: quantification of MBP deposition in airways of challenged animals. Pretreatment with Ab NGF did not decrease MBP deposition in challenged guinea pigs. Ab NGF decreased MBP deposition in atropine-pretreated animals. Values are means $\pm$ SE ($n = 3–5$, with replicates of 5 airways/animal). * $P < 0.05$.



increases eosinophil degranulation 24 h later, as measured by increased MBP deposition in the lungs (Fig. 7) along with decreased eosinophils in airway tissues (Fig. 6). Blocking NGF did not significantly affect eosinophil numbers in the peripheral blood (Fig. 4), bronchoalveolar lavage (Fig. 5), or airways (Fig. 6). Nor did Ab NGF affect eosinophil activation in airways of challenged guinea pigs that were not treated with atropine (Fig. 7E), consistent with no protective effect on airway hyperreactivity (Fig. 1B) or neuronal M_2 receptor function (Fig. 3B). Although Ab NGF did not induce a change in eosinophil numbers, it significantly reduced MBP deposition in atropine-pretreated antigen-challenged guinea pigs (Fig. 7E), coincident with protection from airway hyperreactivity (Fig. 1). Thus atropine-enhanced, antigen challenge-induced hyperreactivity and concomitant increased eosinophil activation in airways are mediated by NGF.

It may be significant that Ab NGF ameliorated vagally induced bronchoconstriction in atropine-treated animals, with a return to control levels (Fig. 1), rather than merely a reduction to challenge levels. This finding illustrates that Ab NGF has a significant protective effect on vagally mediated hyperreactivity,

but only in the context of muscarinic blockade. Since Ab NGF did not protect challenged animals not treated with atropine, we propose that there are both NGF-dependent and -independent pathways leading to vagally mediated hyperreactivity and that atropine changes the mechanism from one that is NGF independent to one that is NGF dependent. This notion is supported by our observation that challenged animals exhibit M_2 receptor dysfunction, whereas challenged guinea pigs treated with atropine do not (Fig. 3). Thus it may be that one pathway involves neuronal M_2 receptor dysfunction but not NGF, whereas the other pathway is NGF dependent and M_2 receptor independent.

The findings presented here differ from a study showing that inhibition of NGF receptors with a tropomyosin-related kinase A (TrkA) antagonist prevents airway hyperreactivity in antigen-challenged guinea pigs (8). One possible explanation for this difference may be the method by which hyperreactivity was measured. In the present study, bronchoconstriction in response to electrical stimulation of the vagus nerves was measured in vagotomized, paralyzed, and ventilated guinea pigs. Thus the experiments described here examine airway

smooth muscle contraction that is mediated by the parasympathetic nerves. By contrast, de Vries et al. (8) measured smooth muscle contraction in response to intravenous histamine in nonvagotomized, spontaneously breathing guinea pigs. It is known that histamine (4) and methacholine (50) induce reflex bronchoconstriction via the vagus nerves. It is therefore reasonable to assume that de Vries et al. were measuring reflex bronchoconstriction mediated by sensory and parasympathetic nerves, whereas the data described here reflect hyperreactivity mediated by parasympathetic nerves. Thus, de Vries et al. could have inhibited TrkA-mediated increases in sensory nerve activity or neuropeptide release, whereas inhibition of NGF in the present study changed parasympathetic activity; neither of these findings is mutually exclusive.

Another important difference between our study and those of de Vries et al. (7–9) is the method employed to inhibit NGF. There are two NGF receptors: the higher-affinity TrkA receptor and the lower-affinity receptor p75 (p75NTR). The relative contribution of these two receptor types to asthma is not completely clear, although airway hyperreactivity has been associated with either receptor (16, 31, 46). It may also be significant that TrkA and p75NTR are expressed by eosinophils (37) and parasympathetic nerves (28), both of which are potential targets for NGF. In the present study, we inhibited NGF with a neutralizing antibody, thereby preventing NGF interactions with TrkA and p75NTR receptors. It may be that inhibiting TrkA, but not p75NTR (8), prevents antigen-induced airway hyperreactivity, whereas blocking NGF interactions with both receptors has an entirely different effect. Similarly, if different NGF receptors mediate the early and late response to allergen, we propose that our findings may be entirely consistent with those of de Vries et al.

NGF may directly regulate eosinophil activation. This idea is supported by *in vitro* studies demonstrating that NGF receptors on eosinophils promote survival (37), chemotaxis, activation and degranulation (45). *In vivo*, the number of eosinophils in bronchoalveolar lavage is higher after antigen challenge in transgenic mice overexpressing NGF in the lung compared with wild-type mice (42). Our data support a model in which NGF, whether released from eosinophils or from other cells (15, 26, 41), can enhance eosinophil activation under conditions of antigen challenge, but that this capacity is normally inhibited by a muscarinic receptor of unknown subtype (M_2R in Fig. 8). Thus parasympathetic nerves can attenuate eosinophil activation under conditions of antigen challenge by limiting the ability of NGF to enhance degranulation. This scenario would explain why atropine enhances eosinophil degranulation in antigen-challenged animals (48) and our finding that Ab NGF is only protective in the presence of atropine (whereby muscarinic inhibition of NGF is removed; Fig. 7).

Although our data indicate the NGF is critical, the source of NGF in this pathway remains unknown. There are many possible sources within the airways, since it has been shown that, in human nasal tissue, NGF localizes in a variety of cell types, including nasal epithelium, submucosal glands, nerves, T lymphocytes, mast cells, and especially eosinophils (51). Thus it may be that eosinophils are a critical source of NGF (45); however, we cannot exclude the possibility that NGF may indirectly affect eosinophil activation via another cell type not identified in the present study (dashed line in Fig. 8). Numerous cells within the lungs, including neuronal cells, inflamma-

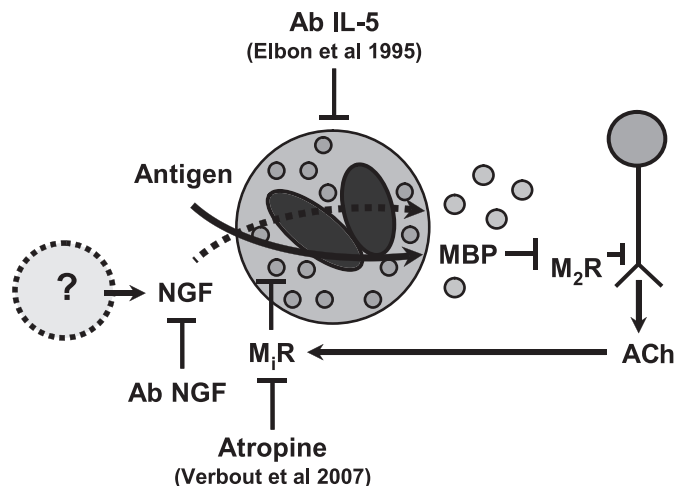


Fig. 8. Antigen challenge releases eosinophil MBP onto neuronal M_2 receptors on airway parasympathetic nerves, causing M_2 receptor dysfunction. Loss of M_2 receptor function increases ACh release onto airway smooth muscle and increases bronchoconstriction (solid arrows and lines). Airway hyperreactivity and neuronal M_2 receptor dysfunction are protected by depletion of eosinophils with antibody to IL-5 (Ab IL-5). NGF does not play a role in this pathway, since Ab NGF does not prevent antigen-induced airway hyperreactivity. In contrast, NGF, the source of which is unknown (?), becomes important when muscarinic receptors are blocked. Muscarinic blockade with atropine at the time of antigen challenge increases eosinophil activation and granule protein release, leading to airway hyperreactivity that is not mediated by M_2 receptor dysfunction (dashed lines). Depletion of eosinophils (Ab IL-5) or inhibition of NGF (Ab NGF) decreases eosinophil activation and prevents atropine-enhanced antigen-induced airway hyperreactivity. One possible mechanism involves a negative regulatory pathway that is controlled by ACh. Inhibitory muscarinic receptors (M_2R) limit NGF and, subsequently, inhibit release of an eosinophil factor. This factor (which may be NGF or another neuromediator) induces a change in parasympathetic nerve activity leading to hyperreactivity through a mechanism that does not involve neuronal M_2 receptor dysfunction.

tory cells, fibroblasts, and smooth muscle cells, express NGF receptors (16), and any of these cells may be part of this pathway.

It is possible that NGF directly increases parasympathetic nerve function via a pathway that is separate from eosinophil MBP release. In guinea pigs, direct application of NGF to airway parasympathetic nerves increases the amplitude of nicotinic fast excitatory postsynaptic potentials, which are blocked by a TrkA antagonist, strongly implicating NGF (28). Additionally, NGF has been demonstrated to have longer-lasting effects on nerve function, via increased expression of neuropeptides (33, 44), including substance P (8, 9, 43, 47), and increased activity or expression of nonspecific cation channels (3, 30). Alternatively, eosinophil-derived NGF may enhance the cholinergic phenotype. Eosinophils increase choline acetyltransferase, vesicular acetylcholine transferase, and acetylcholine content in isolated cholinergic nerves (11). Thus atropine-enhanced, antigen challenge-induced hyperreactivity is mediated by NGF, which appears to increase eosinophil degranulation; however, NGF may also have direct effects on the airway nerves.

Thus interactions between eosinophils and nerves are governed by a balance of inhibitory pathways (Fig. 8). Acetylcholine release is inhibited by M_2 receptors on parasympathetic nerves (1, 18, 21, 35), which are similarly inhibited by MBP (increasing acetylcholine release). Simultaneously, eosinophil activation in antigen-challenged animals is limited by inhibitory muscarinic receptors, which suppress excitatory NGF

pathways. Our data suggest that anticholinergic drugs may promote neurotrophin-mediated inflammation. Elucidation of the type of muscarinic receptor involved in promoting eosinophil activation and airway hyperreactivity, as well as the role of NGF in this effect, will allow development of new therapeutic strategies in asthma.

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