Atropine pretreatment enhances airway hyperreactivity in antigen-challenged guinea pigs through an eosinophil-dependent mechanism

Norah G. Verbout,1 Jesse K. Lorton,1 David B. Jacoby,1,2 and Allison D. Fryer1,2

1Department of Physiology and Pharmacology, 2Division of Pulmonary and Critical Care Medicine, Oregon Health and Science University, Portland, Oregon

Submitted 17 November 2006; accepted in final form 10 January 2007

Verbout NG, Lorton JK, Jacoby DB, Fryer AD. Atropine pretreatment enhances airway hyperreactivity in antigen-challenged guinea pigs through an eosinophil-dependent mechanism. Am J Physiol Lung Cell Mol Physiol 292: L1126–L1135, 2007. First published January 12, 2007; doi:10.1152/ajplung.00455.2006.—Airway hyperreactivity in antigen-challenged animals is mediated by eosinophil major basic protein (MBP) that blocks inhibitory M2 muscarinic receptors on parasympathetic nerves, increasing acetylcholine release onto M3 muscarinic receptors on airway smooth muscle. Acutely, anticholinergics block hyperreactivity in antigen-challenged animals and reverse asthma exacerbations in the human, but are less effective in chronic asthma. We tested whether atropine, given before antigen challenge, affected hyperreactivity, M2 receptor function, eosinophil accumulation, and activation. Sensitized guinea pigs received atropine (1 mg/kg ip) 1 h before challenge and 6 h later. Twenty-four hours after challenge, animals were anesthetized, vagotomized, paralyzed, and ventilated. Airway reactivity to electrical stimulation of the vagi and to intravenous acetylcholine was not altered by atropine pretreatment in nonsensitized animals, indicating that atropine was no longer blocking postjunctional muscarinic receptors. Antigen challenge induced airway hyperreactivity to vagal stimulation that was significantly potentiated by atropine pretreatment. Bronchoconstriction induced by acetylcholine was not changed by antigen challenge or by atropine pretreatment. M2 receptor function was lost in challenged animals but protected by atropine pretreatment. Eosinophils in bronchoalveolar lavage and within airway tissues were significantly increased by challenge but significantly reduced by atropine pretreatment. However, extracellular MBP in challenged airways was significantly reduced by atropine pretreatment, which may account for reduced eosinophils. Depleting eosinophils with antibody to IL-5 before challenge prevented hyperreactivity and significantly reduced MBP in airways of atropine-pretreated animals. Thus atropine pretreatment potentiated airway hyperreactivity by increasing eosinophil activation and degranulation. These data suggest that anticholinergics enhance eosinophil interactions with airway nerves.

anticholinergic; asthma; muscarinic receptors; parasympathetic nerves

PARASYMPATHETIC CONTROL of airway smooth muscle is clearly abnormal in patients with asthma (8). Similarly, in animal models of asthma, hyperreactivity is mediated by increased release of acetylcholine from the parasympathetic nerves onto M3 muscarinic receptors on airway smooth muscle, resulting in increased bronchoconstriction (9, 21, 28, 45). A major mechanism of airway hyperreactivity is the blockade, by eosinophil proteins, of M2 muscarinic receptors on the parasympathetic nerves (10, 11, 16, 19, 21). These neuronal receptors normally limit release of acetylcholine. Loss of M2 receptor function increases acetylcholine release and potentiates vagally induced bronchoconstriction in animals (16, 21) and in humans (1, 46). Anticholinergic medications would therefore be expected to be highly effective treatments for asthma.

Anticholinergic drugs are used clinically to reverse acute bronchoconstriction (59, 60, 70). During acute asthma, adding anticholinergic therapy to β-agonists rapidly improves pulmonary function, decreases rates of hospitalization by 50% (55), and decreases the duration of hospital stay by more than 1 day (3). However, use of anticholinergics in management of chronic stable asthma has been disappointing (4, 30, 31, 35, 69) and is not recommended by the National Institutes of Health Expert Panel on the Management of Asthma (48). This may in part be due to substantial underdosing, coupled with the nonselective nature of the muscarinic antagonists (67). Inhalation of the muscarinic antagonist ipratropium bromide blocks postjunctional M3 muscarinic receptors on airway smooth muscle, but also blocks neuronal M2 receptors, thereby increasing acetylcholine release and potentially overcoming partial blockade of M3 receptors. Indeed, ipratropium does potentiate vagally induced bronchoconstriction in experimental animals via this mechanism (18). Thus addition of anticholinergic drugs has the potential to make airway reactivity worse or at least cancel out the beneficial effects of postjunctional blockade. Alternatively, the poor performance of anticholinergics in chronic asthma may have to do with previously unrecognized effects of anticholinergics on the inflammatory response in the airways.

Eosinophil inflammation of the lungs is a dominant feature of asthma (24, 34, 56) and is associated with airway hyperactivity in experimental animals (10, 41, 44). Eosinophils are recruited to airway nerves via CC-motif chemokine receptor 3 (CCR3) agonists (20), adhere to them via ICAM-1 and VCAM (49, 58), and release eosinophil major basic protein (MBP), an endogenous and selective antagonist for M2 muscarinic receptors (36). Thus, in antigen-challenged guinea pigs, airway hyperreactivity is prevented by depleting eosinophils with antibody to IL-5 (Ab IL-5) (10), by blocking the CCR3 receptors (20), or by blocking eosinophil MBP (11). We used this well-characterized model of airway hyperreactivity, antigen-challenged guinea pigs (17), to test whether the anticholinergic drug atropine would affect development of airway hyperreactivity and eosinophil activation if administered prophylactically.

METHODS

Animals. Specific pathogen-free female Dunkin-Hartley guinea pigs (100–350 g) were purchased from Elm Hill Breeding Labs.
(Chelmsford, MA). Animals 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 United States Animal Welfare Act 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). Three weeks after the last ovalbumin injection, some sensitized animals were challenged with an aerosol of 2.5% ovalbumin for 5 min or until signs of respiratory distress appeared, in which case antigen challenge was immediately halted.

Treatments. Guinea pigs were pretreated 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 rat monoclonal Ab IL-5 (240 µg/kg ip, BD Pharmingen) was administered 4 days before the first injection of ovalbumin.

Measurement of postjunctional muscarinic receptor function. Experiments were conducted 1 day after antigen challenge, by which time atropine had worn off. Guinea pigs were anesthetized with urethane (1.9 g/kg ip, Sigma-Aldrich). This dose produces a deep anesthesia lasting 8–10 h (29), 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 µg·kg⁻¹·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.

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. Atropine (1 mg/kg iv) was given at the end of each experiment to confirm that vagally induced bronchoconstriction was mediated by cholinergic nerves.

Measurement of postjunctional muscarinic receptor function. In the lungs, responsiveness of airway smooth muscle muscarinic receptors was measured. Increasing doses of acetylcholine (1–10 µg/kg iv, Sigma-Aldrich) caused a dose-dependent increase in bronchoconstriction and bradycardia in the heart. The vagus nerves were cut to eliminate acetylcholine-induced reflex responses (66).

Neuronal M2 receptor function. Animals were pretreated with guanethidine (2 mg/kg iv, Sigma-Aldrich) to deplete norepinephrine. Twenty minutes after guanethidine, vagi were stimulated electrically (2 Hz, 0.2-ms pulse duration, for 22 s at 40-s intervals) with voltage adjusted to elicit reproducible bronchoconstrictions between 20 and 40 mmH₂O before the muscarinic agonist pilocarpine (Sigma-Aldrich) was administered, and the effects on vagally induced bronchoconstriction were measured. Pilocarpine stimulates M2 receptors and thus cumulative doses of pilocarpine (1–100 µg/kg iv) inhibit vagally induced bronchoconstriction in a dose-dependent manner. The degree to which pilocarpine inhibits vagally mediated bronchoconstriction is a measure of neuronal M2 receptor function.

Bronchoalveolar lavage. After physiological measurements were made, the lungs were lavaged with five aliquots of 10 ml warm PBS containing 100 µM 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.

Histological evaluation of eosinophils. At the end of the experiments, animals were given 2,500 units of intravenous heparin (American Pharmaceutical Partners) and were exsanguinated by perfusing the jugular vein with warm saline while draining blood 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 in adjacent tissue sections using a mouse monoclonal antibody against protein gene product 9.5 (PGP 9.5) (Biogenesis) as previously described (7). 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 and diluted 1:1,000 in 10% normal goat serum in PBS. Tissue sections were quenched with 3% H₂O₂ in methanol, blocked with CAS-Block (Zymed), and incubated for 90 min at 37°C with a secondary antibody, biotinylated goat anti-mouse IgG (Vector Laboratories) at 1:400 diluted in 5% normal goat serum. Slides were washed with PBS and incubated with an avidin-linked HRP substrate (ABC Elite, Vector Laboratories). PGP 9.5 staining was visualized by incubating tissues with the chromagen diaminobenzidine and nickel (Vector Laboratories), which stained airway nerves black. Eosinophils were visualized by counterstaining with a 1% solution of Chromotrope 2R (Sigma-Aldrich) for 45 min, 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 four to six different cartilaginous airways per animal was counted, using three to five animals per treatment group. 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). The 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. In addition, eosinophils within 8 µm of an airway nerve (approximately the diameter of 1 eosinophil) were also counted. Thus the number of eosinophils per mm² could be calculated for each treatment group, and the proportion of eosinophils associated or not associated with airway nerves could be determined.

Total MBP deposition in lungs. Eosinophil MBP in lung sections was detected using a rabbit monoclonal antibody against guinea pig MBP (43). Lungs were fixed, embedded, sectioned, dewaxed, treated with antigen unmasking solution, and blocked as above. Slides were incubated at 4°C for 24 h with antibody to MBP, diluted 1:1,000 in goat serum, and then incubated for 90 min at 37°C with goat anti-rabbit IgG (Molecular Probes, labeled with Alexa Fluor 594). Slides were washed, mounted under aqueous media with DAPI (Vector Laboratories), and stored at 4°C in the dark. Negative control slides were treated as above without primary antibody.

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 MetaMorph imaging software. The airway lamina propria, smooth muscle, and any outer connective tissue attached or continuous with the smooth muscle were included in the analysis. Airway epithelium, cartilage, blood vessels, and alveoli were excluded.

MBP deposition was quantified using a technique adapted from Tudor et al. (64). Fluorescence intensity in a 10-µm², noncellular region of the lumen was measured in every airway. These values were averaged to obtain the mean background fluorescence, which was then subtracted from each image. MBP signal intensity was thresholded by measuring nonspecific staining in a negative control (absence of
primary antibody) using the tissue with the strongest MBP signal. A serial section from this MBP-containing airway was stained without the primary antibody, and the average fluorescence was measured. This value was used to set the lower limit of the calibration scale for all subsequent measurements. Intensity values above this negative control were included in the analysis; values below this threshold were excluded as nonspecific. Thus only fluorescence above background and separate from nonspecific MBP staining was measured.

The total area of each airway was measured in μm². The area of positive signal above threshold within this area was measured, as was the mean fluorescent intensity. Total intensity was calculated by multiplying the mean fluorescent intensity by the area of positive signal. Average fluorescence per μm² of each airway was determined by dividing total intensity by total area of the airway. Data are means of four to five animals per group, with replicates of four to five airways per animal.

**Distribution of MBP in lungs.** Airways were stained for MBP and photographed in their entirety as described above. Mean fluorescence intensity across the epithelium, lamina propria, smooth muscle, and outer connective/fibrous tissue of each airway was measured using the linescan tool in MetaMorph. Each airway was sampled with four linescans, each consisting of three parallel lines within a 10-μm-wide region. The four linescans were separated by 90° (relative to the center of the airway lumen). Each linescan was drawn from the lumen to the fibrous tissues, and the intensity of each pixel along that line was recorded. Data were sorted by anatomic region: epithelium, lamina propria, smooth muscle, and fibrous tissue, and the average fluorescence intensity per pixel within each anatomic region was calculated. Empty spaces were excluded. Background fluorescence and thresholding were calculated and subtracted as described above. Fluorescence intensity per pixel was converted to fluorescence intensity per μm. Data are mean of four animals per group, with replicates of four airways per animal, and 12 linescan measurements per airway.

**Data analysis and statistics.** All data are expressed as means ± SE. In vivo, the whole frequency response and dose-response curves to nerve stimulation, acetylcholine, and pilocarpine were compared using two-way ANOVA for repeated measures. Histological examination for tissue eosinophils, lavage leukocytes, and MBP content in the airways was analyzed by one-way ANOVA with Fisher’s least significant differences post hoc correction. A P value of less than 0.05 was considered significant. Statistical analyses were made with Kaleidagraph (version 4.01, Synergy Software) or StatView 4.5 (Abacus Concepts).

**RESULTS**

**Atropine pretreatment potentiated airway hyperreactivity in antigen-challenged animals.** Administration of atropine (1 mg/kg ip) 1 h before and 6 h after antigen challenge of guinea pigs did not alter resting pulmonary inflation pressure in any of the groups 24 h later, by which time atropine had worn off (Table 1). In nonsensitized guinea pigs, electrical stimulation of both vagus nerves increased bronchoconstriction in a frequency-dependent manner that was not changed by atropine pretreatment (Fig. 1A). Vagally induced bronchoconstriction was not different in sensitized (not challenged) guinea pigs, regardless of whether or not they were pretreated with atropine (data not shown). However, vagally induced bronchoconstriction was significantly increased 1 day after antigen challenge of sensitized guinea pigs compared with nonsensitized controls (Fig. 1). In contrast to nonsensitized guinea pigs, atropine pretreatment further potentiated vagally induced bronchoconstriction in antigen-sensitized and challenged animals (Fig. 1B). Acetylcholine-induced bronchoconstriction was not altered by antigen challenge or by atropine, demonstrating that atropine had worn off by this time (Fig. 2A). Thus atropine pretreatment exacerbates vagally mediated hyperreactivity in antigen-challenged guinea pigs.

![Image](https://example.com/image.png)

**Fig. 1.** Atropine pretreatment at the time of antigen challenge potentiated airway hyperreactivity in guinea pigs 24 h later. Electrical stimulation of both vagi caused frequency-dependent bronchoconstriction (measured as an increase in pulmonary inflation pressure, mmH₂O) in nonsensitized guinea pigs (A, ○) that was potentiated by antigen challenge (B, □). Atropine pretreatment 1 h before antigen challenge potentiated vagally induced bronchoconstriction in sensitized, challenged (Sens-Challenged) animals (B, ■), but not in nonsensitized controls (A, ●). *The entire frequency response is significantly different from respective frequency response in nonsensitized controls; ‡significantly different from challenged, saline treated. Data are expressed as means ± SE, n = 4–8.

---

**Table 1. Baseline cardiovascular and pulmonary parameters**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>n</th>
<th>Heart Rate (beats/min)</th>
<th>Blood Pressure (mmHg)</th>
<th>Pulmonary Inflation Pressure (mmH₂O)</th>
<th>Bronchoalveolar Lavage Leukocytes (× 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Systolic</td>
<td>Diastolic</td>
<td></td>
</tr>
<tr>
<td>Saline Nonsensitized</td>
<td>8</td>
<td>320.0 ± 9.6</td>
<td>46.6 ± 2.3</td>
<td>24.0 ± 1.8</td>
<td>100.0 ± 6.5</td>
<td>9.1 ± 1.9</td>
</tr>
<tr>
<td>Sensitized</td>
<td>4</td>
<td>308.8 ± 5.2</td>
<td>44.5 ± 5.6</td>
<td>19.5 ± 3.2</td>
<td>97.5 ± 8.5</td>
<td>10.9 ± 1.6</td>
</tr>
<tr>
<td>Sensitized/Challenged</td>
<td>8</td>
<td>315.6 ± 7.8</td>
<td>47.6 ± 2.9</td>
<td>23.7 ± 2.1</td>
<td>85.5 ± 4.1</td>
<td>11.9 ± 1.8</td>
</tr>
<tr>
<td>Atropine Nonsensitized</td>
<td>6</td>
<td>304.2 ± 7.5</td>
<td>43.8 ± 2.7</td>
<td>19.0 ± 3.2</td>
<td>95.7 ± 6.1</td>
<td>13.0 ± 0.9</td>
</tr>
<tr>
<td>Sensitized</td>
<td>5</td>
<td>293.3 ± 6.2</td>
<td>44.4 ± 2.2</td>
<td>21.7 ± 1.6</td>
<td>100.0 ± 7.1</td>
<td>12.0 ± 1.9</td>
</tr>
<tr>
<td>Sensitized/Challenged</td>
<td>6</td>
<td>313.8 ± 16.3</td>
<td>46.5 ± 2.9</td>
<td>23.5 ± 3.3</td>
<td>93.1 ± 6.4</td>
<td>13.5 ± 1.2</td>
</tr>
<tr>
<td>Sensitized/Challenged + Ab IL-5</td>
<td>6</td>
<td>313.3 ± 4.4</td>
<td>44.7 ± 2.4</td>
<td>22.3 ± 2.3</td>
<td>104.2 ± 5.5</td>
<td>10.8 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. Pretreatment with atropine did not change resting heart rate, blood pressure, pulmonary inflation pressure, or total bronchoalveolar lavage leukocytes among groups 24 h after antigen challenge.
Atropine pretreatment did not change vagally mediated bradycardia. Atropine pretreatment did not change resting heart rate or blood pressure in any of the groups tested (Table 1). Electrical stimulation of both vagi resulted in frequency-dependent bradycardia in all groups. Vagally induced bradycardia was increased slightly but not significantly in the antigen-challenged groups (Fig. 3). This effect was not mediated by changes in the postjunctional muscarinic receptors on cardiac muscle since acetylcholine-induced bradycardia was not different among groups (Fig. 2B). Atropine pretreatment had no effect on vagally or acetylcholine-induced bradycardia in either nonsensitized or sensitized and challenged groups, demonstrating again that atropine had worn off by this time.

Ab IL-5 prevented airway hyperreactivity in atropine-pretreated, antigen-challenged animals. To test the role of eosinophils in atropine-induced potentiation of airway hyperreactivity, guinea pigs were treated with Ab IL-5 before antigen sensitization. Ab IL-5 prevented antigen-induced hyperreactivity in atropine-pretreated animals (Fig. 4A) without altering acetylcholine-induced bronchoconstriction (data not shown). Ab IL-5 had no effect on vagally (Fig. 4B) or acetylcholine-induced (data not shown) bradycardia. Thus atropine pretreatment did not potentiate bronchoconstriction in Ab IL-5-treated animals.

Atropine pretreatment prevented M2 muscarinic receptor dysfunction in antigen-challenged animals. In nonsensitized guinea pigs, stimulating M2 receptors with pilocarpine inhibited vagally induced bronchoconstriction in a dose-dependent manner that was not affected by pretreatment with atropine (Fig. 5A), demonstrating functional M2 receptors. In contrast, the M2 receptors did not respond to agonists in antigen-challenged animals, since pilocarpine did not inhibit vagally induced bronchoconstriction (Fig. 5B). Pretreatment with atropine protected neuronal M2 receptor function in antigen-challenged animals (Fig. 5B); this effect was not changed by reducing eosinophils with Ab IL-5 (Fig. 5B). Thus it appears that the function of neuronal M2 receptors does not contribute to atropine-induced potentiation of airway hyperreactivity in antigen-challenged guinea pigs.

**Effect of atropine pretreatment on pulmonary inflammation in antigen-challenged guinea pigs.** We assessed inflammation in the bronchoalveolar lavage and in airway tissues. The total number of inflammatory cells in bronchoalveolar lavage was not different among groups and was not changed by atropine pretreatment (Table 1). However, antigen challenge significantly increased the number of eosinophils in the lavage fluid, an effect that was no longer present in animals pretreated with

**Fig. 2.** In vagotomized guinea pigs, acetylcholine-induced bronchoconstriction, measured as an increase in pulmonary inflation pressure (A, ○) and bradycardia, measured as a fall in heart rate (B, ○), were not changed by atropine pretreatment (closed symbols) or by antigen challenge (squares). Data are expressed as means ± SE, n = 4–8.

**Fig. 3.** Electrical stimulation of both vagi caused a frequency-dependent fall in heart rate, measured in beats/min (A, ○) that was not changed by atropine (●) given 24 h previously. Vagally induced bradycardia was not significantly changed by antigen challenge (●) or by atropine pretreatment (■). Data are expressed as means ± SE, n = 4–8.

**Fig. 4.** Electrical stimulation of the vagus caused bronchoconstriction (A) in animals treated with atropine 24 h previously (●). Antigen challenge significantly potentiated vagally induced bronchoconstriction (■) that was blocked by treatment with antibody to IL-5 (gray diamonds). In the heart, vagally induced bradycardia (B, ●) was not significantly altered by antigen challenge (■) or by antibody to IL-5 (gray diamonds). *The entire frequency response is significantly different from frequency response in nonsensitized controls; ‡significantly different from sensitized challenged plus atropine. Data are means ± SE, n = 4–8. Some data are from Figs. 1 and 3 and reproduced here.
responding to agonists. In contrast, M2 receptor dysfunction was prevented by atropine (Fig. 6). Neither antigen challenge nor atropine pretreatment significantly altered any other inflammatory cell type in the bronchoalveolar lavage fluid (Fig. 6). Thus atropine pretreatment blocked antigen challenge-induced eosinophilia in bronchoalveolar lavage fluid.

Quantitative analysis of eosinophils within the lungs also demonstrated that antigen challenge increased eosinophils in the airways and around nerves (Figs. 7 and 8). As in the bronchoalveolar lavage, atropine pretreatment decreased eosinophils in airways and around nerves of sensitized and challenged animals. Thus while atropine pretreatment increased airway hyperreactivity in antigen-challenged guinea pigs, it significantly decreased eosinophils in bronchoalveolar lavage, in the airway tissues, and around airway nerves.

Analysis of eosinophil activation in the lungs. We measured deposition of eosinophil MBP as an indicator of eosinophil activation in the lungs. Antigen challenge increased, although not significantly, MBP deposition throughout the airways (Fig. 9, A vs. B; quantification in Fig. 9E). Atropine pretreatment before antigen challenge significantly increased MBP deposition within airways of antigen-challenged guinea pigs (Fig. 9, B vs. D; quantification in Fig. 9E), but had no effect in nonsensitized controls (Fig. 9, A vs. C; quantification in Fig. 9E). Increased deposition of MBP throughout airways of atropine-pretreated and antigen-challenged animals was prevented by Ab IL-5 (Fig. 9F; quantification in Fig. 9E).

It appeared that the majority of MBP was located in the region of the lamina propria. Regional analysis of MBP content in the airways confirmed that regardless of treatment, most of the MBP was localized to the lamina propria (Fig. 10). However, regardless of whether MBP was measured in the epithelium, lamina propria, smooth muscle, or fibrous tissue, antigen challenge of sensitized animals with atropine pretreatment significantly increased MBP compared with all other groups (Fig. 10F). There was also a significant increase in MBP in the fibrous tissue region of antigen-challenged animals, and atropine pretreatment increased this further. It is worth noting that this region is where most of the nerve trunks are located (see Fig. 7). Thus eosinophils were more activated in the presence of atropine in antigen-challenged guinea pigs than in nonsensitized animals, and antigen challenge significantly increased MBP in regions of the lung that contain nerves.

DISCUSSION

The data presented in this paper demonstrate that blocking muscarinic receptors with atropine at the time of antigen challenge potentiates challenge-induced airway hyperreactivity 24 h later, by which time atropine had worn off. The ability of atropine to potentiate airway hyperreactivity was specific to antigen-challenged animals, was not present in nonsensitized controls or in sensitized but not challenged animals, and was unrelated to neuronal M3 receptor function. Atropine-induced potentiation was mediated at the level of the parasympathetic nerves supplying the airways, since acetylcholine-induced bronchoconstriction was not potentiated by atropine pretreatment.

Pharmacological blockade of muscarinic receptors by atropine was no longer apparent 24 h later, since neither baseline pulmonary inflation pressure nor baseline heart rate was different between groups (Table 1). Furthermore, neither acetylcholine-induced bronchoconstriction nor bradycardia in atropine-pretreated animals was different from controls at this time point (Fig. 3). Thus, 24 h after atropine administration, the postjunctional M3 muscarinic receptors in the lung and the postjunctional M2 muscarinic receptors in the heart appeared to be fully functional.

During these experiments, nine sensitized animals died during antigen challenge. We have used this antigen challenge protocol extensively, and death is normally a very rare event. It may be significant that of the nine animals that died, eight of them were pretreated with atropine, supporting our observation...
that atropine pretreatment is not beneficial. Histological examination of the lungs demonstrated acute bronchoconstriction in these animals. None of the animals that died during antigen challenge was included in final data analysis.

Airway hyperreactivity 24 h after antigen challenge is vagally mediated (16, 21). This has been confirmed here since vagally induced bronchoconstriction was increased by antigen challenge (Fig. 1), whereas acetylcholine-induced bronchoconstriction in vagotomized animals was not increased (Fig. 2). The potentiating effect of atropine pretreatment on vagal hyperreactivity in antigen-challenged animals was limited to the airways because vagally induced bradycardia was not altered by atropine pretreatment.

Dysfunction of neuronal M2 muscarinic receptors causes vagally mediated hyperreactivity in antigen-challenged animals since the negative feedback these receptors normally provide over acetylcholine release is absent (10, 11, 21). Consistent with our previous studies, neuronal M2 muscarinic receptors were dysfunctional in antigen-challenged guinea pigs because they did not respond to the muscarinic agonist pilocarpine (Fig. 5). However, neuronal M2 receptor function was protected by atropine pretreatment since pilocarpine decreased vagally induced bronchoconstriction in antigen-challenged animals. These findings demonstrate that atropine-induced potentiation of vagally mediated hyperreactivity is not linked to neuronal M2 receptor function.

The mechanism by which atropine pretreatment prevented M2 receptor dysfunction is not known. As we have previously shown that eosinophil MBP binds to M2 receptors (36) blocking their function, it may be that occupancy of the M2 receptors by atropine at the time of antigen challenge prevented MBP from interacting with M2 receptors. Although atropine wears off by 24 h, MBP may not be able to bind to M2 receptors since MBP is highly cationic (25, 52) and therefore unlikely to travel far after its release by eosinophils. Histology demonstrated that MBP was not uniformly deposited in the lungs but was found in aggregates (Figs. 9 and 10), probably at the sites of eosinophil activation.

Increased eosinophils are positively correlated with airway hyperreactivity in humans (2). The role of eosinophils in airway hyperreactivity in antigen-challenged guinea pigs and other experimental animals has been well established (10, 41, 44, 47, 51). Eosinophils cluster along airway nerves in antigen-challenged animals and in patients with fatal asthma (6, 7, 12). When activated, they release several preformed mediators, including eosinophil MBP, eosinophil peroxidase, eosinophil-derived neurotoxin, and neurotrophins including nerve growth factor (38, 61). The dominant protein is MBP (26), which is an
allosteric antagonist of M₂ muscarinic receptors (36) in airways of challenged guinea pigs (7, 42, 53) and in patients with asthma (7, 14, 68), causing airway hyperreactivity by blocking neuronal M₂ receptors (11, 12). Depletion or inhibition of eosinophil migration to the lungs or blockade of eosinophil MBP protects M₂ receptor function and prevents airway hyperreactivity in antigen-challenged animals (10, 11, 15).

Consistent with previous studies (7, 12, 20), we found that antigen challenge increased eosinophils in bronchoalveolar fluid (Fig. 6), in airway tissues, and around airway nerves (Figs. 7 and 8). Although atropine treatment before antigen challenge potentiated airway hyperreactivity, it prevented the antigen-induced eosinophil increase in both bronchoalveolar lavage and within the airways. Nonetheless, eosinophil activation, as assessed by MBP deposition, was increased by atropine in these airways. The mechanism of atropine-induced potentiation of airway hyperreactivity in challenged guinea pigs is linked to the presence of eosinophils, since this effect is abolished by pretreatment with Ab IL-5 (Fig. 4). This illustrates that neither lavage nor histological analysis of eosinophil presence is sufficient to determine the role of eosinophils in the absence of a measure of eosinophil activation.

IL-5 is chemotactic for eosinophils, activates eosinophils, prolongs eosinophil survival, and enhances eosinophil degranulation (23). Ab IL-5 decreases eosinophils in peripheral circulation, inhibits eosinophil migration to the lungs (5, 32), and blocks airway hyperreactivity in antigen-challenged animals (10, 44). In these experiments, Ab IL-5 inhibited atropine-induced potentiation of airway hyperreactivity regardless of whether it was administered before (Fig. 4) or after (data not shown) sensitization. Although Ab IL-5 did not decrease eosinophils in bronchoalveolar lavage, possibly because it was given before sensitization, it clearly prevented eosinophil activation (Fig. 9).

Quantification of MBP revealed significantly greater deposition throughout airways of atropine-pretreated, challenged guinea pigs than in any other group (Figs. 9 and 10). Regardless of treatment group, distribution of MBP was greatest in the airway lamina propria, which contains heparin as a structural scaffold.

Fig. 8. Atropine pretreatment decreased eosinophils in lungs (A) and around nerves (B) of antigen-challenged animals. In saline-pretreated animals (A and B, left), antigen challenge (black bars) significantly increased eosinophils in airways (A) and around nerves (B) compared with respective nonsensitized controls (white bars) and with respective sensitized animals (gray bars). Pretreatment with atropine prevented the antigen-induced increase in eosinophils in airways (A) and around nerves (B). Animals treated with antibody to IL-5 before sensitization were not different from control. Data are expressed as the number of eosinophils per mm² of airway smooth muscle (A) and the number of eosinophils within 8 μm of an airway nerve per mm² (B). *Significantly different from nonsensitized control; ‡significantly different from challenged, saline treated. Data are expressed as means ± SE, n = 3–5.

Fig. 9. Atropine pretreatment increased eosinophil major basic protein (MBP) deposition, labeled with antibody to MBP (red) within airways (A–D and F; increased magnification of area marked with * is shown in insets) of antigen-challenged guinea pigs (nuclei are stained blue with DAPI). Airways of control guinea pigs contain some MBP (A), which was increased by antigen challenge (B). Atropine pretreatment did not increase MBP deposition in nonsensitized controls (C), but significantly increased MBP in challenged animals (D, quantification of data in E). Antibody to IL-5 prevented this increase in MBP deposition in challenged animals pretreated with atropine (F). Data (E) are expressed as mean fluorescence intensity in U/μm² and are means ± SE, n = 3–5. In E, *significantly different from nonsensitized atropine treated (white bar, atropine group); ‡significantly different from challenged, saline treated (black bar, saline group). Scale bar, 100 μm; for insets, scale bar, 30 μm.
component of the basement membrane (71). Since MBP is positively charged (27), it can interact with the negatively charged heparin (36, 62), which may explain the localization in the airway lamina propria. MBP was also significantly increased in the fibrous tissue both by antigen challenge alone and by atropine pretreatment. This may be important because eosinophils accumulate around airway nerves (7, 12, 20), which are found in this region. Thus, atropine pretreatment increased eosinophil activation during antigen challenge, which likely accounts for decreased eosinophil numbers in the lungs.

Eosinophil activation is a better determinant of airway hyperreactivity than eosinophil presence alone. In primates, although antigen-induced hyperreactivity is associated with decreased eosinophils, it is positively correlated with extracellular eosinophil peroxidase in bronchoalveolar lavage, demonstrating eosinophil activation (33). In a clinical trial, an Ab IL-5 did not decrease airway hyperreactivity (40), consistent with it decreasing neither eosinophils nor MBP deposition in the lungs (13), despite depletion of peripheral eosinophils (40). Eosinophil peroxidase is also increased in the urine of animals and humans during acute asthma exacerbations (47, 51), suggesting that eosinophil activation, rather than eosinophil number, may better correlate with clinical status.

Thus atropine pretreatment potentiated antigen-induced vagal hyperreactivity and increased eosinophil MBP in the airways, which may be the result of increased eosinophil activation. The neuronal M2 muscarinic receptor was not involved in atropine potentiation of antigen-induced hyperreactivity, possibly because atropine blocked or protected neuronal M2 receptors during antigen challenge. Without neuronal M2 receptor dysfunction, the mechanism of eosinophil-mediated atropine potentiation of antigen-induced hyperreactivity is unknown.

Eosinophils degranulate and release a variety of neurotrophins, including nerve growth factor (61), leukemia inhibitory factor (72), and brain-derived neurotrophic factor and neurotrophin-3 (50), which may have either direct or indirect effects
on parasympathetic nerves. It is possible that some of these neurotrophins may alter parasympathetic nerve function. Alternatively, eosinophils near airway nerves are activated by chemotactic factors such as eotaxin (20, 37) and by ICAM-1 and VCAM expressed by these nerves (58). Neurotransmitters, such as substance P, can also activate eosinophils (39). There are no known endogenous controls to limit eosinophil activation at the parasympathetic nerves. Mucaricinic receptors are present on inflammatory cells including lymphocytes, macrophages, and eosinophils (22, 54, 57, 63, 65). Should these mucaricinic receptors on eosinophils limit activation, blockade by atropine would remove these inhibitory mechanisms, increasing eosinophil activation and increasing eosinophil interactions with airway nerves.

In summary, pretreatment with atropine, a nonselective anticholinergic drug, potentiated vagally mediated hyperreactivity, an effect that was only observed in antigen-challenged animals. The ability of atropine to potentiate airway hyperactivity was dependent on the presence of eosinophils, since Ab IL-5 prevented both vagally mediated hyperreactivity and the concomitant increase in eosinophil activation. However, atropine pretreatment caused airway hyperreactivity through a mechanism independent from eosinophil MBP-mediated M2 receptor dysfunction.

The data presented here demonstrate that anticholinergic drugs enhance eosinophil activation, which may worsen asthma. This finding may explain the lower-than-expected clinical efficacy of anticholinergic drugs in chronic treatment of asthma. Thus the effects of anticholinergics on inflammatory cells in the airways warrant further investigation and may provide new drug targets.

ACKNOWLEDGMENTS

We thank Kirsten Blensly for the contribution in airway histology analysis and acknowledge Gerald Gleich for providing the antibody to MBP.

GRANTS

This work was supported by American Heart Association predoctoral Fellowship 0515486Z (N. G. Verbout), National Institutes of Health Grants and acknowledge Gerald Gleich for providing the antibody to MBP.

REFERENCES