

Pretreatment with Antibody to Eosinophil Major Basic Protein Prevents Hyperresponsiveness by Protecting Neuronal M₂ Muscarinic Receptors in Antigen-challenged Guinea Pigs

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Abstract

In antigen-challenged guinea pigs there is recruitment of eosinophils into the lungs and to airway nerves, decreased function of inhibitory M₂ muscarinic autoreceptors on parasympathetic nerves in the lungs, and airway hyperresponsiveness. A rabbit antibody to guinea pig eosinophil major basic protein was used to determine whether M₂ muscarinic receptor dysfunction, and the subsequent hyperresponsiveness, are due to antagonism of the M₂ receptor by eosinophil major basic protein. Guinea pigs were sensitized, challenged with ovalbumin and hyperresponsiveness, and M₂ receptor function tested 24 h later with the muscarinic agonist pilocarpine. Antigen-challenged guinea pigs were hyperresponsive to electrical stimulation of the vagus nerves compared with controls. Likewise, loss of M₂ receptor function was demonstrated since the agonist pilocarpine inhibited vagally-induced bronchoconstriction in control but not challenged animals. Pretreatment with rabbit antibody to guinea pig eosinophil major basic protein prevented hyperresponsiveness, and protected M₂ receptor function in the antigen-challenged animals without inhibiting eosinophil accumulation in the lungs or around the nerves. Thus, hyperresponsiveness is a result of inhibition of neuronal M₂ muscarinic receptor function by eosinophil major basic protein in antigen-challenged guinea pigs. (*J. Clin. Invest.* 1997. 100:2254–2262.) Key words: muscarinic receptors • airway hyperresponsiveness • antigen challenge • eosinophils • major basic protein • parasympathetic nerves

Introduction

In the lungs, the dominant autonomic control of airway smooth muscle is provided by the parasympathetic nerves which run in the vagi (1). These nerves release acetylcholine, which causes bronchoconstriction by stimulating postsynaptic M₃ muscarinic receptors on airway smooth muscle (2). In sensitized animals and humans, antigen exposure causes an immediate bronchoconstriction followed by a period of hyper-

responsiveness, characterized by abnormally increased bronchoconstriction to a variety of stimuli (3–5). Atropine blocks this hyperresponsiveness in humans (6, 7) and animals (7), indicating that it is mediated at least in part by the parasympathetic nerves. Hyperresponsiveness is not due to alterations in the function of the M₃ muscarinic receptors since bronchoconstriction in response to exogenously administered acetylcholine remains unaltered following antigen challenge (8). Rather, hyperresponsiveness appears to be due to the increased release of acetylcholine from the parasympathetic nerves (9–11).

Release of acetylcholine from the parasympathetic nerves in the lungs is controlled by neuronal M₂ muscarinic receptors. These neuronal autoreceptors function to inhibit release of acetylcholine from the parasympathetic nerves when stimulated by agonists (12, 13). Therefore, blockade of these autoreceptors with selective muscarinic receptor antagonists increases vagally-induced bronchoconstriction by increasing acetylcholine release. Neuronal M₂ muscarinic receptors are present in most animals species studied (12, 14, 15) and are also present in human lungs (16).

The neuronal M₂ muscarinic receptors on the parasympathetic nerves are dysfunctional in antigen-challenged guinea pigs (8) and in humans with asthma (17, 18). Decreased neuronal M₂ muscarinic receptor function results in increased release of acetylcholine and increased bronchoconstriction. Thus, loss of M₂ muscarinic receptor function could be a mechanism for antigen-induced hyperresponsiveness.

The late phase asthmatic response to antigen is characterized by an influx of eosinophils into the lungs (3, 19–21). Eosinophils reach peak levels 18–24 h after challenge in bronchoalveolar lavage fluid and can still be found in lavage 48 h after antigen challenge (4, 22). After antigen challenge of guinea pigs, eosinophils in the lungs are increased specifically in and around nerve bundles (23, 24). In addition, eosinophil accumulation around nerves is also seen in histological sections from humans who have died from acute asthma (24).

Previous studies have shown that depletion of eosinophils using an antibody to IL-5 inhibits hyperresponsiveness in antigen-challenged guinea pigs (25) and monkeys (26), possibly through protection of neuronal M₂ muscarinic receptor function (23). M₂ receptor function is also protected after repeated antigen challenge by inhibiting eosinophil migration into the lung with an antibody to the eosinophil adhesion molecule very late activation antigen-4 (VLA-4; 27).¹ VLA-4 inhibition prevents eosinophils from adhering to the vascular endothelium, thereby preventing their recruitment to the airways and

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Received for publication 1 May 1997 and accepted in revised form 27 August 1997.

The Journal of Clinical Investigation
Volume 100, Number 9, November 1997, 2254–2262
<http://www.jci.org>

1. Abbreviations used in this paper: MBP, major basic protein; PGP, protein gene product; P_{pit}, pulmonary inflation pressure; VLA-4, very late activation antigen.

airway nerves. Therefore, the recruitment to and presence of eosinophils in the lungs in response to antigen exposure is necessary for loss of neuronal M_2 muscarinic receptor function and for subsequent airway hyperresponsiveness in vivo.

Activated eosinophils release cationic proteins from cytosolic granules (28, 29). One of these proteins, human eosinophil major basic protein (30), is an allosteric antagonist of guinea pig and human M_2 muscarinic receptors in vitro (31, 32). In vivo, neutralization of cationic proteins with anionic substances, including heparin and poly-L-glutamate, acutely restores neuronal M_2 muscarinic receptor function in vivo suggesting that M_2 muscarinic receptors are blocked by a cationic substance (33). Since the cationic eosinophil major basic protein is an antagonist of the M_2 muscarinic receptor in vitro, it may be responsible for decreased neuronal M_2 muscarinic receptor function and thus for antigen-induced hyperresponsiveness in vivo. Using a rabbit antibody to guinea pig eosinophil major basic protein (Ab-MBP; 34), the role of eosinophil MBP in airway hyperresponsiveness and in the loss of the neuronal M_2 muscarinic receptor function was investigated in vivo.

Methods

Animals. Specific pathogen-free female Dunkin-Hartley guinea pigs (250–350 g; supplied by Hilltop Animal Farms, Scottsdale, PA) were used. All animals were shipped in filtered crates and kept in high efficiency particulate-filtered air. Guinea pigs were fed a normal diet (ProLab; Agway, Inc., Syracuse, NY) and were handled in accordance with the standards established by the USA Animal Welfare Acts set forth in National Institute of Health guidelines and the "Policy and Procedures Manual" published by the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee.

Sensitization and challenge with antigen. Guinea pigs were injected intraperitoneally with 10 mg·kg⁻¹ ovalbumin (0.3 ml) every other day for a total of three injections. 3 wk after the last ovalbumin injection, experimental animals were challenged with an aerosol of 5.0% ovalbumin for a maximum of 5 min or until signs of respiratory distress appeared, in which case antigen challenge was immediately stopped. Some animals were injected with rabbit anti-guinea pig eosinophil MBP (Ab-MBP; 1.0 ml i.p. antibody containing rabbit serum; 34) or rabbit serum (1.0 ml i.p.) 18–24 h before antigen challenge. In some antigen-challenged guinea pigs, the Ab-MBP was administered, intravenously or intraperitoneally, 18–24 h after challenge, and the effect on vagally-induced bronchoconstriction was monitored for 1 h after administration.

Sensitization and subsequent challenge were confirmed in antigen-exposed animals by injecting 1.0 ml of 2.5% ovalbumin intravenously. This dose caused acute, lethal anaphylaxis in antigen-challenged, Ab-MBP-pretreated, and serum-pretreated guinea pigs, but this dose had no effect in control guinea pigs.

Measurements of pulmonary inflation pressure. Experiments were conducted 18–24 h after exposure to ovalbumin. The guinea pigs were anesthetized with urethane (1.5 g·kg⁻¹, i.p.) This dose produces a deep anesthesia lasting 8–10 h (35), although none of these experiments lasted for longer than 3 h.

Both jugular veins were cannulated for the administration of drugs. One internal carotid artery was cannulated for measurement of blood pressure using a DTXTM pressure transducer (Viggo-Spectramed, Oxnard, CA), and the heart rate was derived from the blood pressure tracing using a tachograph. The trachea was cannulated and the animals were ventilated with a positive pressure, constant volume rodent respirator (Harvard Apparatus, Inc., South Natick, MA) at a tidal volume of 1 ml·kg⁻¹ and a respiratory rate of 100 breaths per minute. The animals were paralyzed by infusing succinylcholine (10

μg·kg⁻¹·min⁻¹, i.v.) Pulmonary inflation pressure (P_{pi}) was measured at the trachea using a DTXTM pressure transducer (Viggo-Spectramed). A positive pressure of 70–130 mm H₂O (mean 99.3 ± 2.4 mm H₂O) was needed to ventilate the animals. All signals were recorded on a polygraph (Grass Instrument Co., Quincy, MA). Bronchoconstriction was measured as the increase in P_{pi} above the basal inflation pressure produced by the ventilator (36). The sensitivity of the method was increased by taking the output P_{pi} signal from the driver of one channel to the input of the preamplifier of a different channel on the polygraph. Thus, baseline P_{pi} was recorded on one channel and increases in P_{pi} above the baseline were recorded on a separate channel. With this method, increases in P_{pi} as small as 2–3 mm H₂O can be accurately recorded. All animals were pretreated with guanethidine (5 mg·kg⁻¹, i.p.) This dose of guanethidine has been demonstrated to deplete norepinephrine (14), and produces a temporary reduction in the magnitude of vagally-induced bronchoconstriction and bradycardia.

Studies of vagal hyperresponsiveness. Anesthetized and ventilated animals were paralyzed with succinylcholine (10 μg·kg⁻¹·min⁻¹ i.v.) Both vagus nerves were cut and the distal ends were placed on stimulating electrodes. Electrical stimulation of both vagus nerves produced bronchoconstriction and bradycardia. The vagus nerves were stimulated at frequencies ranging from 2.0–15.0 Hz for 5 s at 90-s intervals keeping both pulse duration (0.2 ms) and voltage (10.0 V) constant among groups. Changes in P_{pi} were recorded on a Grass polygraph as described above.

Studies of neuronal M_2 muscarinic receptor function. Nerve stimulation was conducted at 50-s intervals on anesthetized, ventilated, and paralyzed guinea pigs. The effects of agonists are more apparent at lower frequencies of stimulation, thus the effects of pilocarpine on vagally-induced bronchoconstriction were tested using 2.0 Hz (12). Pulse duration (0.2 ms) and the stimulus train (45 pulses per train) were kept constant. 30 min after guanethidine and before administration of pilocarpine, baseline responses to electrical stimulation of the vagus nerves were obtained. The voltage was chosen at the beginning of each experiment (within a range of 2.5–45.0 V; mean 13.9 ± 2.8 V) to give a mean bronchoconstriction of 22.3 ± 0.8 mm H₂O. Cumulative doses of pilocarpine (1–100 μg·kg⁻¹, i.v.) were administered and the effect on vagally-induced bronchoconstriction was measured. The results are expressed as a ratio of bronchoconstriction in the presence of pilocarpine to the bronchoconstriction in the absence of pilocarpine.

30–100 μg·kg⁻¹ of pilocarpine produced a small, transient bronchoconstriction. Therefore, the effect of these doses of pilocarpine on vagally-induced bronchoconstriction was measured after the P_{pi} had returned to baseline.

Studies of M_3 muscarinic receptor function. After the pilocarpine dose response and frequency response experiments, the function of the M_3 muscarinic receptors on airway smooth muscle was tested with increasing doses of acetylcholine (1–8 μg·kg⁻¹, i.v.).

Bronchoalveolar lavage. At the end of the experiment, bronchoalveolar lavage was performed via the tracheal cannula. The lungs were lavaged with five aliquots of 10.0 ml PBS. Guinea pigs tested for sensitization and challenge with 1.0 ml of 2.5% ovalbumin were not lavaged because of decreased lung volume and increased pulmonary resistance. The recovered lavage fluid (35–45 ml) was centrifuged. In order to remove any erythrocytes, the cells were resuspended in 10 ml of deionized water and an additional 40 ml of PBS was added to stabilize the remaining cells. Cells were centrifuged again and resuspended in 10 ml of PBS. Total cells were counted under a Neubauer Hemocytometer (Hausser Scientific, Hoarsham, PA). Aliquots of the cell suspension were cytospun onto glass slides, stained with Diff-Quik[®] (Baxter Healthcare Corp., McGraw Park, IL) and counted.

Pathological evaluation. At the end of each experiment, the lungs were removed, inflated, and fixed with 3.7% formaldehyde in 0.1 M phosphate buffer. 24 h later, the lungs were removed from the fixative and rinsed with 0.1 M phosphate buffer. Transverse sections from the trachea and from each lobe of the lung (3–5 mm in thick-

ness) were taken for pathological evaluation. Sections were embedded in paraffin blocks, sliced in consecutive 6.0 μm sections, and mounted onto glass slides for light microscopy.

Identification of airway nerves using protein gene product (PGP) 9.5. The polyclonal rabbit antibody to PGP 9.5(37) was used to identify airway nerves. Sections of paraffin embedded tissue 6 μm in thickness were dewaxed by immersion in xylene, rehydrated through a graded alcohol series and washed three times in Tris-HCl (pH 7.8). Sections were then placed in a solution of Tissue Unmasking Fluid® at 90.0°C for 10 min and brought to room temperature over 10 min and washed in Tris-HCl. Nonspecific peroxidase was inhibited by applying a solution of 3.0% hydrogen peroxide in methanol for 10 min followed by three 5 min washes with Tris-HCl. The tissue was then overlaid with 10.0% normal goat serum for 30 min and rabbit anti-PGP 9.5 (1:4,000) was applied to the tissue for 48 h at 4.0°C. The tissue was washed three times in Tris-HCl over 15 min and a biotinylated goat anti-rabbit antibody (Vector Laboratories, Inc., Burlingame, CA) was added (1:50) for 30 min. The tissue was rewashed three times over 15 min with Tris-HCl and an avidin horseradish peroxidase complex was added for 30 min. The antibody was detected by the addition of the chromagen SG® in the presence of hydrogen peroxide (pH 5.3). The chromagen SG® stained the nerves black. Rabbit serum absorbed over PGP 9.5 was used as a control antibody. To identify eosinophils, the sections were stained in a solution of 1.0% Chromotrope 2R for 30 min. The sections were then washed in tap water for 5 min, dehydrated in a graded alcohol series, defatted in xylene, and coverslipped.

Endogenous peroxidase activity, as is found in eosinophils and neutrophils, was quenched using methanol and peroxidase. Thus, only airway nerves and not inflammatory cells were detected using the peroxidase detection system with SG® as the chromagen. Eosinophils were subsequently stained with chromotrope 2R. The airways examined histologically were major bronchi and all were of similar size. Major bronchi were studied since these are the site of the greatest cholinergic nerve innervation (38) and also the site of the maximal accumulation of eosinophils around airway nerves (24).

Histological analysis. Four cartilaginous bronchi from each of five animals representing each experimental group were examined (20 airways per group). Airways were selected by starting at the top left corner of the slide and moving in a counter clockwise manner. An obvious landmark within an airway was chosen under low power as the starting point for analysis. Beginning at this point, the sections were viewed under an oil immersion lens, this image was captured to a video camera attached to the microscope and relayed to a viewing screen. The image was oriented so that the basement membrane was uppermost, thus, a section of airway containing submucosa and airway smooth muscle was examined. Ten consecutive sections were examined by two investigators. The number of eosinophils within 8.0 μm of a PGP 9.5 immunoreactive nerve and the total number of eosinophils were counted in each section. The area examined as well as the area of the whole airway wall were calculated using an image analysis program (Image Pro Plus®, Media Cybernetics, L.P., Silver Spring, MD).

Drugs and reagents. Acetylcholine, guanethidine, ovalbumin, PBS, pilocarpine, rabbit serum, sodium chloride, succinylcholine, and urethane were purchased from Sigma Chemical Co. (St. Louis, MO). Purified rabbit anti-guinea pig MBP antibody was the same reagent as described previously (34, 39). All drugs were dissolved and diluted in 0.9% NaCl or PBS. ABC Elite, biotinylated goat anti-rabbit antibody, normal goat serum, and the chromagen SG® were all purchased from Vector Laboratories, Inc. Chromotrope 2R was purchased from Sigma Chemical Co. Tissue Unmasking Fluid® was purchased from Signet Laboratories, Inc. (Dedham, MA). The polyclonal rabbit antibody to PGP 9.5 and control PGP 9.5 immune absorbed serum were purchased from Biogenesis, Inc. (Sandtown, NH).

Statistics. All data are expressed as mean \pm SEM. Acetylcholine, frequency, and pilocarpine responses were analyzed using two-way ANOVA for repeated measures. Baseline heart rates, blood pres-

ures, pulmonary inflation pressures, and changes in pulmonary inflation pressure (before pilocarpine administration), histological measurements, and bronchoalveolar lavage were analyzed using ANOVA (Statview 4.5; Abacus Concepts, Inc., Berkeley, CA, a *P* value of 0.05 was considered significant).

Results

There were no significant differences between the baseline P_{pi} (control, 106 ± 6 ; antigen challenged, 97 ± 4 ; antigen challenged with Ab-MBP, 96 ± 4 ; and antigen challenged with serum, 95 ± 6 mm H₂O) and baseline heart rate (control, 258 ± 13 ; antigen challenged, 266 ± 5 ; antigen challenged with Ab-MBP, 270 ± 8 and antigen challenged with normal serum, 281 ± 6 beats minute^{-1}) among groups.

Electrical stimulation of both vagus nerves caused bronchoconstriction and bradycardia. There were no statistically significant differences in the voltage used between groups. In the absence of pilocarpine the bronchoconstrictions were not significantly different between groups (control, 23.2 ± 2.3 ; antigen challenged, 22.2 ± 1.5 ; antigen challenged with Ab-MBP, 21.0 ± 1.4 ; and antigen challenged with serum 19.9 ± 0.9 mmH₂O).

Neuronal M₂ muscarinic receptor function was tested using the muscarinic agonist pilocarpine. Increasing doses of pilocarpine ($1\text{--}100$ $\mu\text{g}\cdot\text{kg}^{-1}$) inhibited vagally-induced bronchoconstriction in a dose-dependent manner in control guinea pigs. Inhibition of vagally-induced bronchoconstriction was expressed as a ratio of vagally-induced bronchoconstriction in the presence of pilocarpine to vagally-induced bronchoconstriction in the absence of pilocarpine (B2/B1). In control guinea pigs, the maximum dose of pilocarpine, 100 $\mu\text{g}\cdot\text{kg}^{-1}$, inhibited vagally-induced bronchoconstriction by 67% (B2/B1 was 0.33 ± 0.08). However, in the antigen-challenged guinea pigs, pilocarpine did not significantly inhibit vagally induced bronchoconstriction. The maximum effect of pilocarpine, at 100 $\mu\text{g}\cdot\text{kg}^{-1}$, was only a 13% inhibition (B2/B1 ratio of 0.87 ± 0.08). The effect of pilocarpine in these animals was significantly different from controls (Fig. 1).

In antigen-challenged guinea pigs pretreated with Ab-MBP intraperitoneally, 1 h before challenge, pilocarpine did inhibit vagally-induced bronchoconstriction in a dose-related manner (maximum inhibition at 100 $\mu\text{g}\cdot\text{kg}^{-1}$ was 58%; B2/B1 = 0.42 ± 0.07). The dose response to pilocarpine in the Ab-MBP-pretreated animals was significantly different from the challenged animals and was not different from controls (Fig. 1). In a few guinea pigs, administration of Ab-MBP either intraperitoneally ($n = 1$) or intravenously ($n = 2$) 18–24 h after antigen challenge did not inhibit vagally-induced bronchoconstriction, or restore the ability of pilocarpine to inhibit vagally-induced bronchoconstriction in a dose-related manner (data not shown).

In challenged animals pretreated with rabbit serum, pilocarpine did not cause a dose-related inhibition of vagally-induced bronchoconstriction. Maximum inhibition of vagally-induced bronchoconstriction at 100 $\mu\text{g}\cdot\text{kg}^{-1}$ pilocarpine was 22% (B2/B1 ratio = 0.78 ± 0.05). This effect on vagally-induced bronchoconstriction in the normal serum treated animals was not significantly different from the antigen-challenged animals, but it was significantly different from control and Ab-MBP pretreated guinea pigs (Fig. 1).

Vagal hyperresponsiveness was measured by nerve stimu-

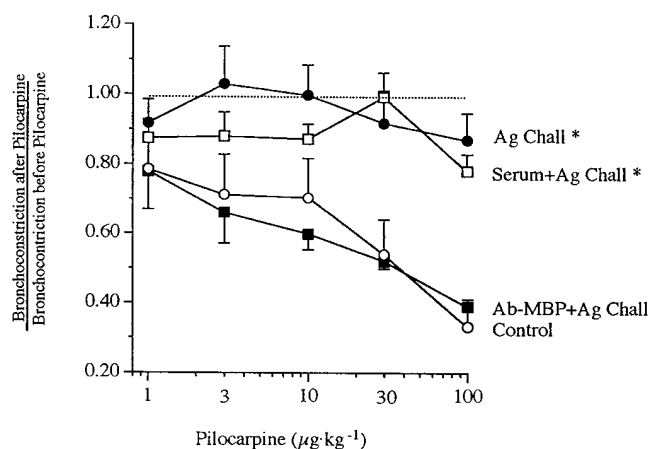


Figure 1. Ab-MBP protects neuronal M_2 muscarinic receptor function in antigen-challenged guinea pigs. Electrical stimulation of cut vagus nerves (2.0 Hz, 0.2 ms, 13.9 ± 2.8 V, 22 s) produced bronchoconstriction. In controls (open circles), pilocarpine (1–100 $\mu\text{g}\cdot\text{kg}^{-1}$, i.v.) inhibited vagally-induced bronchoconstriction. In antigen-challenged guinea pigs (closed circles) pilocarpine did not inhibit vagally-induced bronchoconstriction. Pretreatment with Ab-MBP (1.0 ml, i.p.; closed squares) protected the response to pilocarpine. Data are expressed as the means of ratios of bronchoconstriction after pilocarpine over bronchoconstriction before pilocarpine \pm SEM, $n = 4$ –6 animals. *Signifies statistically significant difference from control and Ab-MBP.

lation. Electrical stimulation of the distal ends of the cut vagus nerves at increasing frequencies (2.0–15.0 Hz, 0.2 ms, 10.0 V, 5 s train, at 90 s intervals) produced frequency-dependent bronchoconstriction, measured as an increase in P_{pi} . Vagally-induced bronchoconstriction was significantly potentiated in antigen-challenged guinea pigs compared with control. In the Ab-MBP

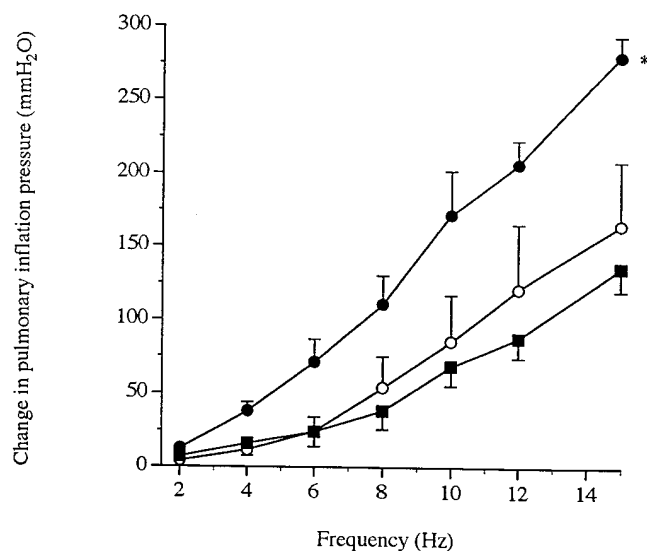


Figure 2. Electrical stimulation of cut vagus nerves (2.0–15.0 Hz, 10.0 V, 0.2 ms, 5 s) produced frequency-dependent bronchoconstriction measured as an increase in P_{pi} . Vagally-induced bronchoconstriction in antigen-challenged guinea pigs (closed circles) was significantly greater than control (open circles) and antigen-challenged Ab-MBP-pretreated (closed squares) guinea pigs (*). Data are expressed as the mean increase in P_{pi} (mm H_2O) \pm SEM; $n = 5$ –6 animals.

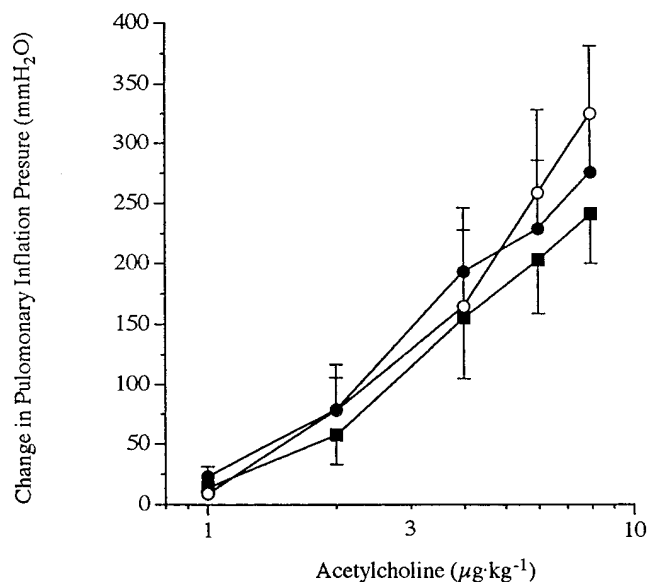


Figure 3. Acetylcholine (1–8 $\mu\text{g}\cdot\text{kg}^{-1}$, i.v.) produced dose-dependent bronchoconstriction in vagotomized guinea pigs. There were no statistically significant differences between control (open circles), antigen-challenged (closed circles), and antigen-challenged Ab-MBP-pretreated (1.0 ml, i.p.; closed squares) guinea pigs. Data are expressed as mean increases in P_{pi} (mm H_2O) \pm SEM; $n = 7$ –8 animals.

pretreated guinea pigs, the frequency dependent bronchoconstriction was not significantly different from control guinea pigs, and it was significantly different from antigen-challenged guinea pigs (Fig. 2).

M_3 muscarinic receptor function on airway smooth muscle was tested using a dose-response curve to acetylcholine in vagotomized guinea pigs. Administration of exogenous acetylcholine (1–8 $\mu\text{g}\cdot\text{kg}^{-1}$, i.v.) induced dose-dependent bronchoconstriction in control, antigen-challenged, and challenged guinea pigs pretreated with Ab-MBP. There were no significant differences among any of these groups (Fig. 3).

Both electrical stimulation of the vagus nerves and intravenous acetylcholine caused bradycardia in addition to the bronchoconstriction. There was a frequency-dependent and dose-dependent increase in bradycardia that was not different between groups. In addition, pilocarpine at 10–100 $\mu\text{g}\cdot\text{kg}^{-1}$, i.v. caused a small transient dose-dependent bradycardia that was not different among groups (data not shown).

Airway leukocyte populations were measured by bronchoalveolar lavage. There was a small, nonsignificant rise in the total number of leukocytes returned in bronchoalveolar lavage from antigen-challenged animals ($15.8 \pm 4.4 \cdot 10^6$ cells) compared with controls ($7.9 \pm 3.1 \cdot 10^6$ cells). Pretreatment with normal serum did not affect the increase in antigen-challenged guinea pigs ($15.5 \pm 6.1 \cdot 10^6$ cells). However, in the guinea pigs pretreated with the Ab-MBP there was a further rise in total cells recovered in the bronchoalveolar lavage above that with antigen challenge alone ($26.1 \pm 7.5 \cdot 10^6$ cells), which was significantly different from control but not from antigen challenge alone and normal serum pretreated. This increase was due to an increase in macrophages (Fig. 4), although the rise in macrophages was not significant. Antigen challenge of non-pretreated, Ab-MBP-pretreated, and normal serum-pretreated

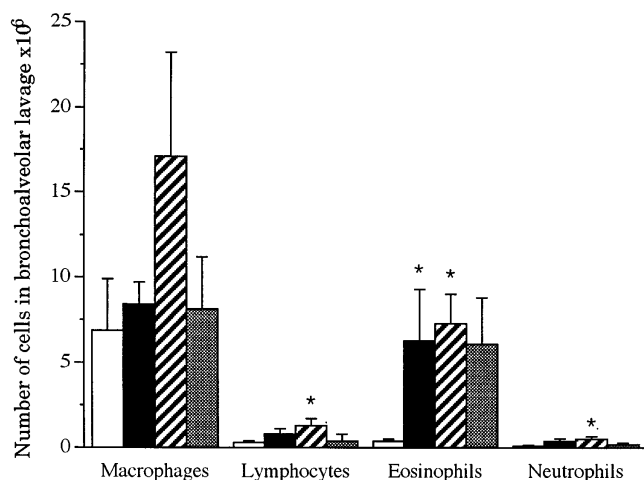


Figure 4. Airway leukocyte populations were measured in bronchoalveolar lavage. There was a significant increase (*) in the number of eosinophils in lavage from antigen-challenged guinea pigs (solid bars; $n = 6$), antigen-challenged guinea pigs pretreated with Ab-MBP (hatched bars; $n = 8$), and antigen-challenged guinea pigs pretreated with rabbit serum (speckled bars; $n = 7$) compared with controls (open bars; $n = 4$). Data are expressed as means of total number of cells retrieved in lavage \pm SEM.

guinea pigs did cause an increase in lymphocytes and neutrophils returned in bronchoalveolar lavage. This increase was statistically significant between Ab-MBP-pretreated and control guinea pigs for both cell types (Fig. 4).

There was a significant increase in the total number of eosinophils returned in bronchoalveolar lavage in the antigen-challenged animals compared with the control animals. In Ab-MBP-pretreated antigen-challenged animals, the number of eosinophils in bronchoalveolar lavage was also significantly greater than in control animals. There was also a marked increase in the number of eosinophils returned in bronchoalveolar lavage from serum pretreated animals. While this increase closely matched those observed in Ab-MBP-pretreated and non-pretreated antigen-challenged guinea pigs, it was not statistically different from controls (Fig. 4).

The airway walls of guinea pigs were examined histologically to assess the relationship of eosinophils to nerves. The mean airway wall area was the same in each of the three groups ($0.42 \pm 0.08 \text{ mm}^2$ in controls, $0.41 \pm 0.05 \text{ mm}^2$ in antigen-challenged animals, and $0.31 \pm 0.05 \text{ mm}^2$ in antigen-sensitized animals pretreated with Ab-MBP). Airway nerves were detected using an antibody to PGP 9.5 and a peroxidase-linked detecting system (Figs. 6–8). Compared with controls, the number of eosinophils in the lungs was increased after antigen challenge as assessed by histology (Figs. 5 A, 6, and 8). In addition, antigen challenge also caused an increase in the number of eosinophils associated with the nerves in the airways (Figs. 5 B and 6). Pretreatment with Ab-MBP did not alter the number of eosinophils in the lungs or the number of eosinophils in association with the airway nerves (Figs. 5 and 7).

Discussion

In anesthetized guinea pigs baseline heart rate, blood pressure, and P_{pi} were not different between control and antigen-chal-

lenged animals. Neither treatment with antibody to eosinophil MBP (Ab-MBP) nor with rabbit serum before antigen challenge affected baseline heart rate, blood pressure, or P_{pi} .

In the heart, a homogenous population of M_2 muscarinic receptors induce bradycardia when stimulated by acetylcholine. Because bradycardia induced either by intravenous acetylcholine or by electrical stimulation of the vagus nerves, did not differ among control, antigen-challenged, or challenged guinea pigs pretreated with serum or with Ab-MBP, the M_2 muscarinic receptors in the heart were not altered by any of these treatments.

In contrast to the lack of effect of antigen challenge on vagally-induced bradycardia, vagally-induced bronchoconstriction in the lungs was significantly potentiated after antigen challenge (Fig. 2), confirming the results of Fryer et al. (27). The function of the M_3 muscarinic receptors on airway smooth muscle was not altered by antigen challenge since dose-response curves to intravenous acetylcholine were not significantly different from each other. Previously, it was reported, using single doses of acetylcholine, that there was a small, though insignificant, increase in acetylcholine-induced bronchoconstriction after antigen challenge (8). However, when a full dose-response curve to acetylcholine was tested, there was no difference between challenged and control animals. Other labs have suggested that antigen challenge increases release of acetylcholine from the vagus nerves in dogs (9), guinea pigs (10), and mice (11). In the absence of any change in the sensitivity of airway smooth muscle to acetylcholine, the data presented here confirm that the increase in vagally-induced bronchoconstriction after antigen challenge must also be due to increased release of acetylcholine from the vagus nerves.

In the lungs, release of acetylcholine from the vagus nerves

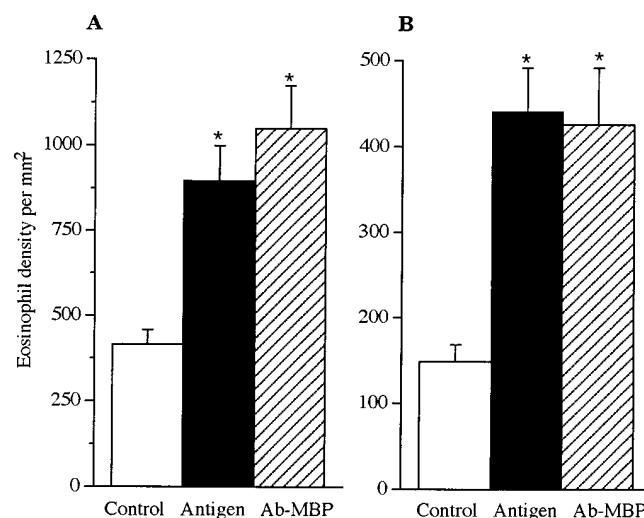


Figure 5. Eosinophil number in the airways (A) and in association with the airway nerves (B) was measured in histologic sections of bronchi from guinea pigs. There were significantly more eosinophils in the airways and in association with the airway nerves of antigen-challenged guinea pigs (solid bars) compared with controls (open bars). This increase in eosinophils in antigen-challenged guinea pigs was not altered by pretreatment with the Ab-MBP (hatched bars). *Significantly different from control. Data are expressed as mean number of eosinophils per $\text{mm}^2 \pm$ SEM; $n = 5-6$ animals.

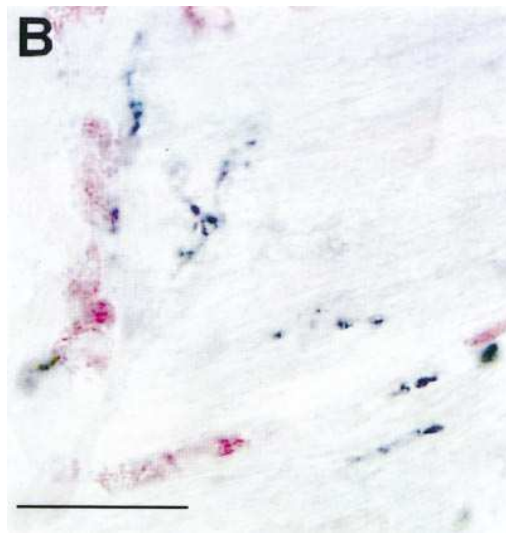
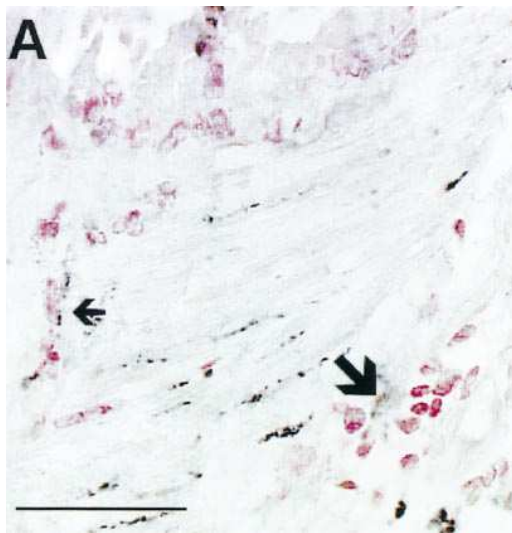


Figure 6. Photomicrograph of a paraffin embedded section of an antigen-challenged guinea pig bronchus. Airway nerves, in black, have been stained using an antibody to PGP 9.5 while eosinophils have been stained red with Chromotrope 2R. Eosinophils were seen in close proximity to and touching the nerves in the airway smooth muscle under low power (*small arrows, A*) and shown under higher magnification in *B*. Eosinophils were also seen accumulating around the airway nerve bundles in the submucosa (*thick arrows*). Bar represents 50 μ m in *A* and 20 μ m in *B*.

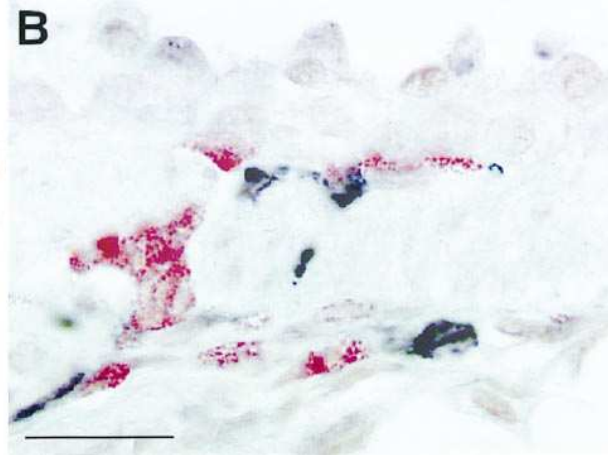
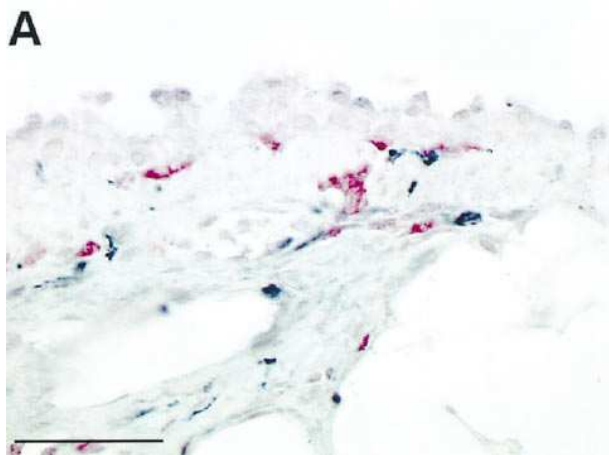


Figure 7. Photomicrograph of a paraffin embedded section of a bronchus from an antigen-challenged guinea pig pretreated with antibody to eosinophil MBP. Airway nerves and eosinophils have been stained as in Fig. 6. Eosinophils were seen in the airways and in association with airway nerves in the airway smooth muscle under low power (*A*) and under higher magnification (*B*). Bar represents 50 μ m in *A* and 20 μ m in *B*.

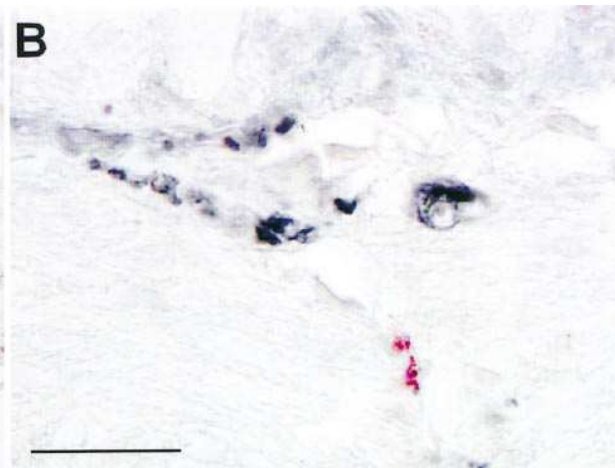
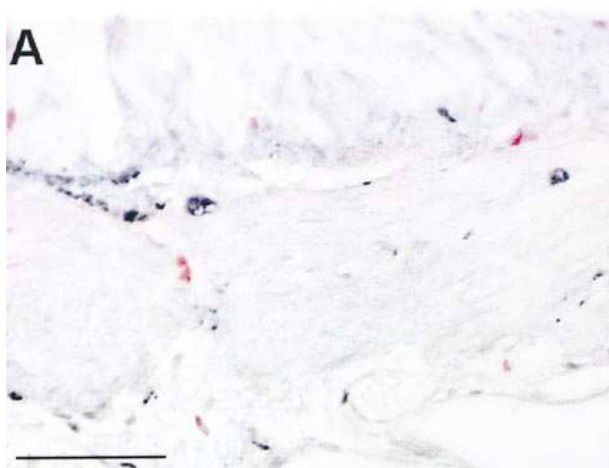


Figure 8. Photomicrograph of a paraffin-embedded section of a control guinea pig bronchus, under low (*A*) and high power (*B*). Airway nerves and eosinophils have been stained as in Fig. 6. Occasional eosinophils were seen in the airway wall and in association with airway nerves. Bar represents 50 μ m in *A* and 20 μ m in *B*.

is limited by neuronal M_2 muscarinic receptors (12). Pharmacological blockade of these neuronal receptors increases release of acetylcholine from these nerves (40, 41). However, after antigen challenge, the neuronal M_2 receptors are dysfunctional since pharmacological agents no longer inhibit or potentiate vagally-induced bronchoconstriction by stimulating or blocking the neuronal M_2 receptors (8). In the experiments reported here, the M_2 receptors were also dysfunctional after antigen challenge since the muscarinic agonist, pilocarpine, was no longer able to inhibit vagally-induced bronchoconstriction.

Function of the neuronal M_2 muscarinic receptors in antigen-challenged guinea pigs is acutely restored by anionic compounds such as heparin and poly-L-glutamate within 20 min of administration (33). Thus, dysfunction of the neuronal M_2 receptors *in vivo* is due to the presence of an endogenous, positively-charged substance that inhibits M_2 muscarinic receptor function. These data also suggest that the neuronal M_2 muscarinic receptors have not been damaged by antigen challenge, but are blocked by an endogenous, cationic antagonist.

A candidate for this M_2 antagonist is eosinophil MBP. Eosinophils are implicated in loss of M_2 receptor function since they are selectively recruited to the airway nerves of antigen-challenged guinea pigs (24). Depletion of eosinophils with an antibody to IL-5 (23), or prevention of eosinophil migration into the lungs with an antibody to VLA-4 (27) protects M_2 muscarinic receptor function in antigen-challenged guinea pigs. The role for eosinophil MBP in loss of M_2 receptor function is further supported by the finding that eosinophil MBP, but not eosinophil cationic protein or eosinophil derived neurotoxin, displaces [3 H]NMS from M_2 receptors, but not from M_3 muscarinic receptors *in vitro*. While eosinophil peroxidase will weakly displace [3 H]NMS from M_2 muscarinic receptors, it has a low affinity and is probably not physiologically relevant (31, 32).

In the studies reported here, pretreatment with Ab-MBP protected the function of the neuronal M_2 muscarinic receptors since the ability of pilocarpine to inhibit vagally-induced bronchoconstriction in the Ab-MBP pretreated, antigen-challenged guinea pigs was identical to controls. This protection was not due to nonspecific factors in the antiserum since normal rabbit serum alone did not prevent loss of M_2 receptor function with antigen challenge. This is in agreement with previous studies showing that isotype-matched antibodies had no effects on neuronal M_2 muscarinic receptor function (27). Protection of the M_2 receptors was not due to inhibition of eosinophil migration into the lungs since eosinophils in the airways, in association with the nerves, and in the bronchoalveolar lavage were not inhibited by pretreatment with the antiserum. Furthermore, recruitment of eosinophils to the nerves was not inhibited by Ab-MBP pretreatment since eosinophil association with the nerves was not significantly altered in histological sections of the airways. Thus, even in the presence of eosinophils in the lungs the function of the neuronal M_2 muscarinic receptors was protected by Ab-MBP, confirming that loss of receptor function in the lungs of antigen-challenged guinea pigs is due to recruitment of eosinophils to the nerves (23, 27) and to the subsequent binding of eosinophil MBP to neuronal M_2 muscarinic receptors.

Although pretreatment of guinea pigs with Ab-MBP protected neuronal M_2 receptor function, giving the antibody intravenously or intraperitoneally after antigen challenge did not restore receptor function. Heparin and poly-L-glutamate were

able to acutely restore receptor function by competing with the M_2 muscarinic receptors for eosinophil major basic protein (33). However, Ab-MBP was unable to do this. The inability of the Ab-MBP to remove bound eosinophil major basic protein from the M_2 muscarinic receptor may be because the specific epitopes are bound to the M_2 muscarinic receptors, and are therefore unavailable to the antibody. Thus, in contrast to the effect of heparin, Ab-MBP may be unable to remove bound MBP from the M_2 receptors.

In antigen-challenged guinea pigs, electrical stimulation of the vagus nerves produced a frequency-dependent bronchoconstriction, which was significantly greater than controls (Fig. 2). Thus, antigen-challenged guinea pigs were hyperresponsive. Pretreatment with Ab-MBP prevented the development of antigen-induced hyperresponsiveness. These data confirm previous studies showing that eosinophil MBP can induce hyperresponsiveness (42–44).

Hyperresponsiveness in guinea pigs is associated with decreased neuronal M_2 muscarinic receptor function (27, 45, 46) and in antigen-challenged guinea pigs with increased eosinophils in the lungs. Protecting M_2 receptor function, by inhibiting eosinophil migration into the lungs, prevented hyperresponsiveness (27). In addition, restoring M_2 muscarinic receptor function acutely with intravenous heparin, which neutralizes cationic proteins such as MBP (47, 48), simultaneously reversed hyperresponsiveness to stimulation of the vagus nerves (33). Finally, protecting neuronal M_2 receptor function with Ab-MBP also prevented vagally-induced hyperresponsiveness. Thus, airway hyperresponsiveness is likely to be the direct result of antagonism of the inhibitory, neuronal M_2 muscarinic receptors by eosinophil MBP in antigen-challenged guinea pigs.

In asthma, hyperresponsiveness has a vagal component, since anticholinergics are effective bronchodilators (7, 49, 50). Dysfunction of the neuronal M_2 muscarinic receptors has been demonstrated in humans with asthma (17, 51) and may contribute to the hyperresponsiveness characteristic of this disease (52). Decreased function of the neuronal M_2 receptors in asthma may also be due to the presence of eosinophil MBP since eosinophils and MBP, which are rarely found in the lungs of nonasthmatics, are increased in the lungs of asthmatics (21, 22, 53). Furthermore, eosinophils and eosinophil MBP are found associated with the nerves in lungs from humans with fatal asthma (24). In guinea pigs, heparin reversed hyperresponsiveness by removing eosinophil MBP and restoring M_2 receptor function (33). In asthmatic humans, inhaled heparin also inhibited antigen-induced hyperresponsiveness (54). Therefore, positively charged substances are associated with hyperresponsiveness in asthmatic patients. Thus, it is possible that in humans, blockade of neuronal M_2 muscarinic receptors by eosinophil MBP may be a mechanism for the hyperresponsiveness experienced in asthma.

Acknowledgments

The authors thank Bethany Yost for technical assistance. The authors also wish to thank Dr. George Jakab for the use of his inhalational facilities and Brian Schofield for his assistance with the histology (both laboratories supported by NIH grant ES-03819).

This work was funded by National Institutes of Health grants HL-44727 and HL-53543 (A.D. Fryer), by AI-37163 and HL-54659 (D.B. Jacoby), by AI-09728 and AI-34577 (G.J. Gleich), by HL-09389

(R.W. Costello), and by the Foundation for Fellows in Asthma Research Award (R.W. Costello) and by the Council for Tobacco Research, Center for Indoor Air Research, and by the American Heart Association.

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