

Molecular mechanisms in allergy and clinical immunology

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Eosinophil recruitment to the airway nerves

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Increased vagal reflexes contribute to bronchoconstriction in asthma. Antigen challenge of sensitized animals induces vagal hyperresponsiveness. This review will discuss the evidence that eosinophils increase release of acetylcholine from the parasympathetic nerves. After antigen challenge, eosinophils are actively recruited to the airway nerves, possibly through expression of chemotactic substances and adhesion molecules by the nerves. Tachykinins acting on neurokinin 1 receptors activate the eosinophils. Activated eosinophils release eosinophil major basic protein (MBP), which is an endogenous antagonist for M₂ muscarinic receptors. The M₂ muscarinic receptors on the parasympathetic nerves in the lungs normally inhibit release of acetylcholine. When M₂ receptors are blocked by MBP, acetylcholine release is increased, resulting in hyperresponsiveness. Neutralization of MBP with polyanionic substances restores M₂ receptor function and eliminates hyperresponsiveness. Antibodies to MBP prevent M₂ receptor dysfunction and hyperresponsiveness, as do antibodies to the adhesion molecule very late antigen 4, which prevent eosinophil migration. A low dose of dexamethasone, which does not affect total eosinophil influx into the lungs and airways, prevents eosinophils from clustering around the nerves and prevents antigen-induced M₂ dysfunction and hyperresponsiveness. Furthermore, animal studies show that viral infections, which are important precipitants of asthma attacks, and exposure to air pollutants such as ozone can also activate airway eosinophils, leading to a chain of events similar to that seen after antigen challenge. Finally, a similar clustering of eosinophils around airway nerves, as well as release of MBP onto the nerves, is seen in fatal asthma, suggesting that similar mechanisms may be involved in human airway hyperresponsiveness. (*J Allergy Clin Immunol* 2001;107:211-8.)

Key words: Muscarinic receptors, asthma, parasympathetic nerves, hyperresponsiveness, bronchoconstriction

Abbreviations used

ICAM: Intracellular adhesion molecule
MBP: Major basic protein
NK: Neurokinin
VCAM: Vascular cell adhesion molecule
VLA: Very late antigen

It has long been recognized that asthma is characterized by infiltration of the airways by eosinophils. Much attention has been paid to eosinophils in the airway epithelium because these are accessible to bronchoscopic biopsy.^{1,2} However, eosinophils are also recruited to the airway nerves in patients with asthma³ (Fig 1). How these eosinophils are recruited and activated and their roles in the pathophysiology of asthma will be the subject of this review.

AIRWAY NERVES

The airways are supplied with a network of nerves that can cause bronchoconstriction or relaxation. In addition to effects on airway smooth muscle, the nerves also affect secretions and vascular permeability and may also be involved in mediating inflammation.

The predominant neural control of the airway smooth muscle is provided by the parasympathetic nervous system (for review see Costello and Fryer⁴). Reflex bronchoconstriction can be elicited by a variety of stimuli in the airways, including sulfur dioxide,⁵ exercise,⁶ cigarette smoke,⁷ histamine,⁸ and cold air.⁹ Furthermore, reflex bronchoconstriction is increased in patients with asthma.^{10,11}

Although some studies have concluded that the cholinergic nerves are not important in allergic asthma,¹²⁻¹⁷ these studies are flawed by the use of single, low doses of an inhaled anticholinergic drug without demonstrating adequate vagal nerve blockade. Anticholinergic agents appear to be poorly adsorbed when given by inhalation. Thus anticholinergic agents are effective bronchodilators when given intravenously but are less effective in studies where they are given by inhalation.^{18,19} Furthermore, currently available anticholinergics are nonselective. Thus atropine and ipratropium may block inhibitory M₂ muscarinic

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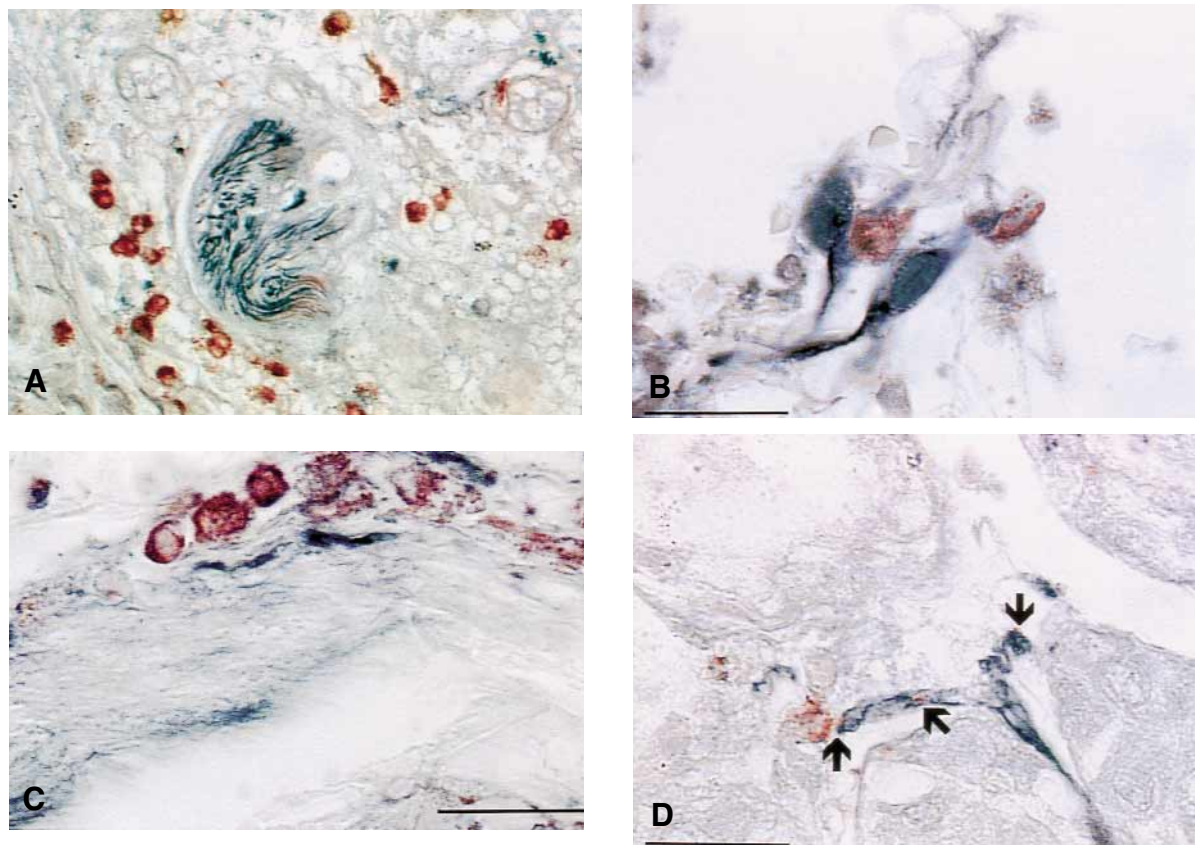


FIG 1. Eosinophils and extracellular MBP are found in association with nerve fibers in the airways of patients with asthma who died during an asthmatic attack. Photomicrographs are from paraffin-embedded sections of asthmatic airways. Eosinophils (detected with an antibody to human MBP, *in red*) are seen in close proximity to airway nerves in the airway smooth muscle (detected with an antibody to PGP 9.5, *in black*). Eosinophils are found around nerve bundles (A), ganglia (B), and nerve fibers (C). Non-cell-associated MBP is found as a red smear on nerve bundles (A) and spotted onto nerve fibers (D). (From Costello RW, et al. *Am J Physiol* 1997;273[1 Pt 1]:L93-103.)

receptors on the nerves (see below) and increase acetylcholine release, opposing the postjunctional blockade of the M_3 muscarinic receptors on airway smooth muscles.²⁰ Despite these problems, high-dose atropine²¹⁻²³ and ipratropium²⁴⁻²⁶ are effective bronchodilators in allergic asthma. Viral infection is also clearly associated with hyperresponsiveness that is vagally mediated.^{27,28} Thus the cholinergic nervous system does play an important role in many forms of asthma.

INHIBITORY MUSCARINIC RECEPTORS ON THE NERVES

In the airways, release of acetylcholine from postganglionic parasympathetic fibers stimulates smooth muscle contraction by binding to M_3 muscarinic receptors on the smooth muscle. However, at the same time, released acetylcholine feeds back onto inhibitory M_2 receptors on the nerve endings themselves (Fig 2). Binding of acetylcholine to these inhibitory autoreceptors provides a negative feedback, decreasing further release of acetylcholine.

The importance of these inhibitory neuronal M_2 receptors becomes apparent when they are blocked with M_2 selective antagonists. Blocking feedback inhibition of acetylcholine release can increase by as much as 10-fold the bronchoconstrictor response to electrical stimulation of the vagus nerve. Conversely, stimulating the M_2 receptors with a muscarinic agonist, such as pilocarpine, can inhibit the bronchoconstrictor response to vagal stimulation by as much as 85%. These neuronal M_2 receptors are present in guinea pigs,²⁹ dogs,³⁰ cats,^{31,32} and rats³³ and also in human beings.³⁴

M_2 RECEPTOR DYSFUNCTION IN HUMAN BEINGS WITH ASTHMA AND IN ANIMAL MODELS OF ASTHMA

In some human beings with asthma, the M_2 receptor is dysfunctional. Minette et al³⁵ demonstrated that in healthy subjects inhalation of the muscarinic agonist pilocarpine stimulates M_2 receptors and inhibits the vagally mediated reflex bronchoconstriction that occurs after subsequent inhalation of sulfur dioxide. This effect

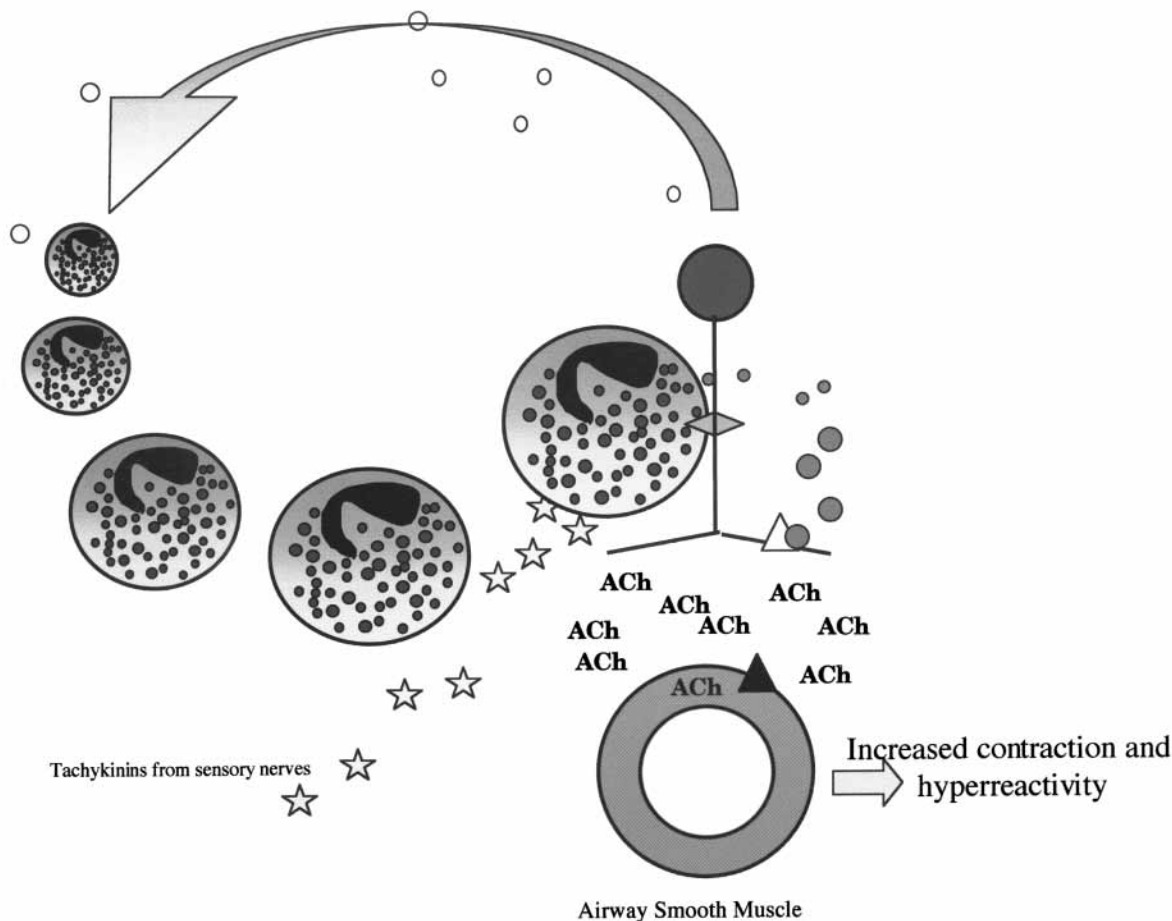


FIG 2. After antigen challenge, eosinophils are actively recruited to the airway nerves, possibly through release of chemotactic substances (arrow and open circles). Eosinophils appear to adhere to the nerves through adhesion molecules (diamonds) and are activated by endogenous tachykinins (stars), acting through NK1 receptors. Activated eosinophils release MBP (solid circles), which blocks the inhibitory M₂ muscarinic receptors on the parasympathetic nerves (open triangle). Loss of M₂ receptor function leads to increased release of acetylcholine (ACh), which binds to M₃ muscarinic receptors on the airway smooth muscle (solid triangle), leading to increased bronchoconstriction.

was not seen in similar experiments in patients with atopic asthma. Evidence of M₂ receptor dysfunction was also found in a study by Ayala and Ahmed.³⁶ However, not all patients with asthma have dysfunctional M₂ receptors. Vagally mediated bronchoconstriction after treatment with propranolol was inhibited by pilocarpine in patients with asthma.³⁷ Whether the difference in M₂ function among patients with asthma reflects different groups with different pathogenic mechanisms or whether this relates to differences in treatment and control of airway inflammation remains to be determined.

We have demonstrated that the inhibitory neuronal M₂ muscarinic receptors are dysfunctional in 3 different animal models of asthma and hyperresponsiveness, viral infection,³⁸ antigen challenge,³⁹ and exposure to ozone.⁴⁰ All of these conditions are associated with increased parasympathetic drive to the airway smooth muscle. In all 3 cases, loss of M₂ receptor function leads to hyperresponsiveness and increased vagally mediated bronchoconstriction.

EOSINOPHILS AND M₂ RECEPTOR DYSFUNCTION

The mechanisms for M₂ receptor dysfunction have been most completely elucidated in the antigen-challenged guinea pig. M₂ receptor dysfunction and vagally mediated hyperresponsiveness are absolutely dependent on an inflammatory response, and the eosinophil is the key inflammatory cell in the setting of antigen challenge. Eosinophils not only invade the airway but are also found in high concentrations in the vicinity of the airway nerves³ (Fig 3), as is seen in patients with asthma (Fig 1).

Effects of blocking eosinophil influx

We treated ovalbumin-sensitized guinea pigs with TRFK-5 (a monoclonal antibody to IL-5) before exposing them to aerosols of ovalbumin as described above. The antibody blocked the influx of eosinophils into the lungs as assessed both histologically and by whole-lung

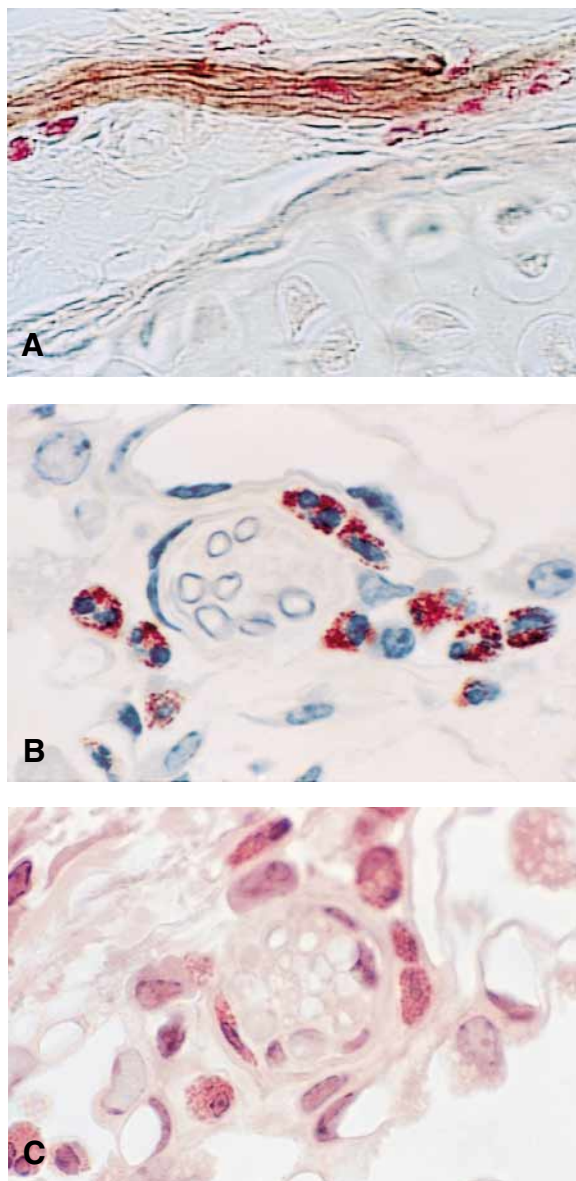


FIG 3. Airway nerves from guinea pigs that have been sensitized and challenged with antigen are surrounded by eosinophils. Paraffin-embedded sections of bronchus from 3 different antigen-challenged guinea pigs are shown. In antigen-challenged guinea pigs, eosinophils surround the nerves (a nerve is shown in lateral view in **A** and in cross section in **B**) and infiltrate the nerve bundles (**C**). **A**, Airway nerve bundles are stained by acetylcholinesterase immunohistochemistry, and eosinophils are stained with eosin. (**B** is stained with Luna stain, and **C** is stained with hematoxylin-eosin) (From Costello RW, et al. *Am J Physiol* 1997;273[1 Pt 1]:L93-103.)

lavage.⁴¹ Although antigen challenge caused loss of M_2 receptor function in untreated guinea pigs, in those guinea pigs that received the IL-5 antibody, M_2 receptor function was preserved.

Another molecule that is important in antigen-induced lung eosinophilia is very late antigen 4 (VLA-4), an adhesion molecule on the eosinophil that binds to vascu-

lar cell adhesion molecule 1 (VCAM-1) on endothelial cells. We demonstrated that a monoclonal antibody to VLA-4 blocked the influx of eosinophils into the lungs in antigen-challenged animals. It also prevented loss of M_2 receptor function and prevented development of hyperresponsiveness, the effects of antigen challenge on the M_2 receptor.⁴² Thus eosinophils are firmly linked to M_2 receptor dysfunction and subsequent hyperresponsiveness in antigen-challenged guinea pigs.

Role of eosinophil proteins

Many M_2 receptor antagonists are positively charged, and the positively charged protein protamine is an M_2 antagonist.⁴³ Three of the granular proteins of the eosinophil, major basic protein (MBP), eosinophil cationic protein, and eosinophil peroxidase, are among the most strongly positively charged mammalian proteins. We postulated that these positively charged proteins might be functioning as endogenous antagonists at the M_2 receptors. We used both *in vivo* studies, in which we attempted to neutralize these positively charged proteins using the polyanionic substances heparin and poly-L-glutamic acid, and receptor-ligand-binding studies to investigate this possibility.

For the heparin and poly-L-glutamic acid study, we determined the response to vagal stimulation in antigen-challenged guinea pigs. This was increased compared with that of control guinea pigs because of M_2 receptor dysfunction. After a stable response to vagal stimulation was obtained, either heparin (2000 U/kg intravenously) or poly-L-glutamic acid (15 mg/kg intravenously) was given, and the responses to vagal stimulation were measured every minute thereafter. The response to vagal stimulation was unchanged for 5 minutes, after which a progressive decrease in vagally mediated bronchoconstriction was observed for 20 minutes, reaching a plateau at approximately 50% of the bronchoconstriction before administration of the heparin or poly-L-glutamic acid (Fig 4). Responses to intravenous acetylcholine were unchanged by heparin or poly-L-glutamic acid, demonstrating that the M_3 receptors on the airway smooth muscle were not affected. However, responses to M_2 receptor agonists and antagonists were restored by these treatments, demonstrating that the reduction in response to vagal stimulation was due to restoration of M_2 receptor function. Neither of the polyanionic substances affected vagally induced bronchoconstriction in non-antigen-challenged animals. A similar effect can be demonstrated with partially desulfated (nonanticoagulant) heparin.⁴⁴ The effect of these substances, structurally very different but all polyanionic and capable of neutralizing eosinophil proteins *in vitro*,⁴⁵ is consistent with the positively charged proteins of the eosinophil being responsible for the loss of M_2 receptor function.

A number of studies in patients with asthma have demonstrated beneficial effects of inhaled heparin.^{46,47} Although many have concentrated on mast cells as the target of the heparin, the results are also consistent with a decrease in reflex bronchoconstriction through the mechanisms described here.

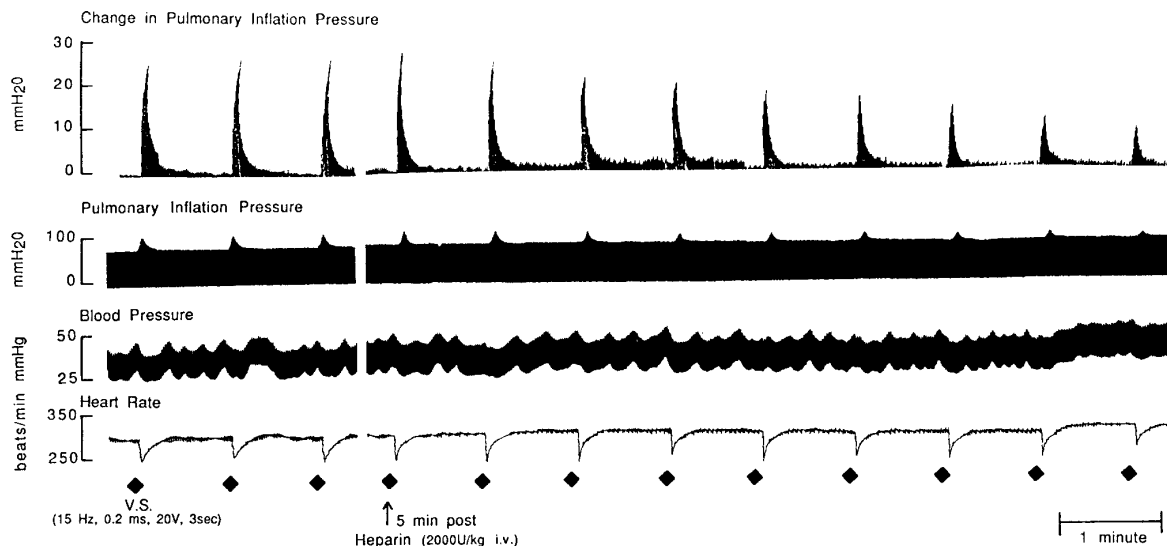


FIG 4. Heparin inhibits vagally induced bronchoconstriction in sensitized guinea pigs challenged with ovalbumin. Electrical stimulation of both vagus nerves (V.S. and at *black diamonds*) causes bradycardia (measured as a fall in heart rate) and bronchoconstriction (measured as a rise in pulmonary inflation pressure). The first 3 responses to vagal nerve stimulation on the *left* are in the absence of heparin. The remaining responses are 6 to 14 minutes after intravenous administration of heparin 2000 U/kg. Starting 6 minutes after heparin administration, vagally induced bronchoconstriction is decreased. In contrast, vagally induced bradycardia is unaltered by heparin. Heparin did not alter either baseline pulmonary inflation pressure or heart rate. (From Fryer AD, Jacoby DB. *J Clin Invest* 1992;90:2292-8.)

We also tested the effect of pretreating guinea pigs with an antibody against guinea pig MBP. This treatment had no effect on the eosinophil influx into the airways after antigen challenge, but it did prevent hyperresponsiveness to vagal stimulation and M_2 receptor dysfunction.⁴⁸

Radioligand-binding studies of human eosinophil proteins

We undertook a series of *in vitro* ligand-binding studies using purified human eosinophil MBP, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin. We found that MBP displaced ^3H -N-methylscopolamine (a muscarinic ligand) from M_2 , but not from M_3 , muscarinic receptors,^{49,50} with a dissociation constant of about 1.5×10^{-5} mol/L. This is within the likely concentration range for this protein in the airways⁵¹ and is therefore likely to be physiologically relevant. We also demonstrated that the effect of MBP on M_2 receptors is an allosteric one (ie, MBP binds to a site different from the primary ligand-binding site and induces a conformational change, decreasing binding at the primary site).

Similar effects were demonstrated with eosinophil peroxidase, although this protein was considerably less potent than MBP. Interestingly, eosinophil cationic protein did not share this property, demonstrating that structural determinants, as yet undefined, other than positive charge are required for a protein to be an M_2 antagonist.⁴⁹ Likewise, the noncationic eosinophil-derived neurotoxin was not an M_2 receptor antagonist.

Thus both *in vivo* and *in vitro* studies point to eosinophil MBP as the mediator that blocks M_2 receptor function.

Eosinophilic inflammation of airway nerves

Histologic sections of the airways of antigen-challenged guinea pigs reveal eosinophils surrounding and infiltrating the nerves (Fig 3). We counted the number of eosinophils per airway nerve in antigen-challenged and in control animals in which the M_2 receptor function had been tested *in vivo* with pilocarpine, which normally suppresses vagally mediated bronchoconstriction by stimulating M_2 receptor.³ The number of eosinophils correlated with the degree of M_2 receptor dysfunction (Fig 5). There was no correlation of M_2 receptor function with either the number of macrophages or the number of lymphocytes in the nerves. Furthermore, the eosinophils appeared to cluster around the nerves, being found in about twice the density around the nerves as they were around the adjacent blood vessels, and substantially higher than in other parts of the airway (Fig 6). Interestingly, eosinophils are also associated with the nerves in the skin of patients with prurigo nodularis⁵² and with nerves in the gastrointestinal tract with chronic inflammation⁵³⁻⁵⁵ and in the optic nerves and spinal cords in mice with experimental allergic encephalomyelitis.⁵⁶

Eosinophils are also found in the airway nerves of patients who died of acute asthma (Fig 1). The association of eosinophils with the nerves can be demonstrated from the airway parasympathetic ganglion (Fig 1, *B*) all the way to the nerve ending (Fig 1, *C* and *D*) (as is also true in the antigen-challenged guinea pig and rat⁵⁷). In addition, costaining sections with antibody to the nerve-specific protein PGP 9.5 and with antibody to human

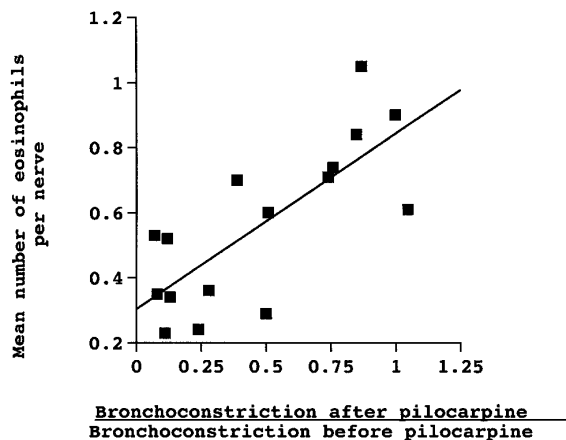


FIG 5. Loss of neuronal M_2 muscarinic receptor function is associated with increased eosinophil levels around the nerves of antigen-challenged guinea pigs. Data are derived from control guinea pigs ($n = 6$), from animals that received a single antigen challenge ($n = 5$), and from animals that received 4 challenges ($n = 5$). The function of the M_2 receptor is expressed as a ratio of the degree of vagally induced bronchoconstriction after 100 $\mu\text{g/kg}$ pilocarpine to that obtained before pilocarpine administration. When the receptor is dysfunctional, this ratio is high because the agonist pilocarpine has no effect on the receptor. In the absence of pilocarpine, the vagally induced bronchoconstriction was not significantly different among the 3 groups. The correlation between the mean number of eosinophils per nerve and the in vivo measurement of the neuronal M_2 receptor function is 0.55 ($P = .001$). (From Costello RW, et al. *Am J Physiol* 1997;273[1 Pt 1]:L93-103.)

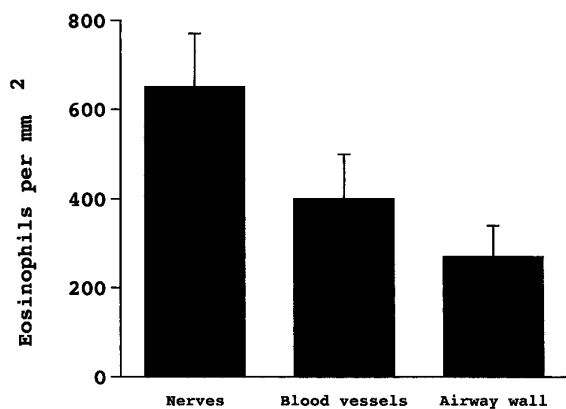


FIG 6. Density of eosinophils within a 15- μm area of the airway nerve, within a similar area around an adjacent blood vessel and within the airway wall (from the basement membrane to serosa, excluding the cartilage). Results are obtained from the analysis of 2 airways from each of 10 antigen-challenged guinea pigs (20 total). The adjacent blood vessels ($<100 \mu\text{m}$ away) were also examined because more than 90% of airway nerves are found in close proximity to a blood vessel; accumulation of eosinophils around the nerves could reflect eosinophils migrating out of the blood vessels. Note the significantly greater density of eosinophils around the airway nerves than in either the blood vessels or the airway wall. (From Costello RW, et al. *Am J Physiol* 1997;273[1 Pt 1]:L93-103.)

eosinophil MBP reveals deposits of extracellular MBP along airway nerves (Fig 1, A and D).

Recruitment of eosinophils to the airway nerves

Although the mechanisms responsible for the recruitment of eosinophils to the airway nerves are incompletely understood, we have demonstrated that primary cultures of airway parasympathetic neurons express eotaxin,⁵⁸ as well as the adhesion molecules intracellular adhesion molecule 1 (ICAM-1)⁵⁹ and VCAM (unpublished data). Eosinophils bind to these neurons in culture, and this interaction can be prevented by an antibody to Mac-1, the counterligand for ICAM-1 (unpublished data).

Expression of ICAM-1 in these cultured nerve cells is inhibited by dexamethasone (unpublished data). This may be significant in that we have recently demonstrated that a low dose of dexamethasone prevents antigen-induced M_2 receptor dysfunction in guinea pigs without blocking the influx of eosinophils into the airways, assessed both histologically and by lung lavage. However, careful histologic examination reveals that whereas the total number of eosinophils in the airway tissues is not decreased by dexamethasone, the influx of eosinophils into the airway nerves is completely blocked.⁶⁰

The mechanisms responsible for eosinophil activation are also incompletely understood. Because tachykinins may play an important role in airway inflammation after antigen inhalation,⁶¹ we studied the effects of neurokinin (NK) receptor antagonists. Pretreating sensitized guinea pigs with CP-96,345, CP-99,994, or SR-140,333 (NK-1 receptor antagonists) but not MEN 10,376 (an NK-2 receptor antagonist) before antigen challenge prevented loss of M_2 receptor function.⁶² However, these treatments did not affect either the total number of eosinophils in the airway or the number of eosinophils around the nerves. Because tachykinins can activate eosinophils in vitro,⁶³ we tested the effect of a stable analogue of substance P on airway M_2 receptor function in pathogen-free guinea pigs.⁶⁴ Intravenous injection of this analogue caused immediate loss of M_2 receptor function that was blocked by antibody to MBP, suggesting that tachykinins may be responsible for eosinophil activation.

Eosinophils in other models of airway disease

Viral infections increase vagal reflex bronchoconstriction in both human beings^{27,28} and experimental animals.⁶⁵ Although the airway M_2 receptor is also dysfunctional in virus-infected guinea pigs,³⁸ eosinophils do not participate in this effect.⁶⁶

Recent studies suggest that most asthma attacks may be caused by viral infections,^{67,68} and yet asthma attacks are associated with eosinophils in the airways. We postulated that this may be due to the atopic background of the patient with asthma causing an eosinophil response to viral infection. To model this, we sensitized guinea pigs to ovalbumin (by intraperitoneal injection) but did not challenge them. Infecting these guinea pigs with parain-

fluenza virus caused them to develop M₂ receptor dysfunction. However, while virus-induced M₂ dysfunction was not blocked by antibody to MBP in nonsensitized animals, in sensitized animals it was blocked by this antibody and reversed by heparin, demonstrating dependence on eosinophil MBP. Thus viral infections of the airways may recruit and activate eosinophils in the setting of an atopic background.⁶⁶

In this model it may be significant that the eosinophil appears also to have a beneficial effect. Viral titers in the lungs of sensitized animals were more than an order of magnitude lower than those in the lungs of nonsensitized animals. When eosinophils were depleted (with antibody to IL-5), the antiviral effect of sensitization was lost.⁶⁶

Similarly, ozone inhalation causes M₂ receptor dysfunction in guinea pigs⁴⁰ and in human beings.⁶⁹ In the guinea pig, hyperresponsiveness and M₂ receptor dysfunction immediately after ozone are mediated by eosinophils. Depletion of eosinophils with an antibody to IL-5 or blocking of eosinophil MBP with an antibody prevents M₂ receptor dysfunction and hyperresponsiveness. Likewise, removal of MBP with heparin restores M₂ receptor function and reverses hyperresponsiveness.⁷⁰ In this model, however, M₂ receptor function is normal 2 days after exposure to ozone, suggesting that MBP is metabolized within 48 hours. New eosinophils move into the lungs 3 days after ozone exposure, but at this point they appear to have a beneficial effect because blocking them makes hyperresponsiveness worse. Thus after ozone exposure eosinophils have a dual effect, initiating M₂ receptor dysfunction early but participating in the repair process later.

Sensitization changes the inflammatory response to ozone. As in the virus-infected guinea pigs, sensitization to ovalbumin before exposure to ozone switches the mechanism of long-term hyperresponsiveness to be eosinophil and M₂ receptor dependent.⁷¹ Whereas depleting eosinophils makes ozone-induced hyperactivity worse in nonsensitized guinea pigs, it completely prevents hyperactivity in sensitized animals.⁷¹

SUMMARY

We have shown that eosinophils are recruited to the airway nerves in antigen-challenged animals and in human beings with asthma. Antigen challenge causes loss of function of inhibitory M₂ receptors on the nerves. This can be blocked by manipulations that prevent the influx of eosinophils into the lung, specifically antibodies to IL-5 and VLA-4. The recruitment of eosinophils to the nerves may be mediated by chemotactic substances produced by the neurons (such as eotaxin), and binding of eosinophils to the nerves may involve interactions with adhesion molecules (eg, ICAM-1 and VCAM) expressed by the neurons. Activation of eosinophils at this stage is likely mediated by tachykinins, leading to release of eosinophil proteins. Eosinophil MBP acts as an M₂ receptor antagonist, increasing reflex bronchoconstriction. Eosinophils also mediate M₂ receptor dysfunction

in sensitized animals infected with virus and in ozone-exposed animals. In each of these cases, the eosinophil appears also to have a beneficial effect, decreasing viral replication in virus-infected sensitized animals and participating in recovery from ozone-induced hyperresponsiveness.

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