Antibody to VLA-4, but not to L-selectin, protects neuronal M2 muscarinic receptors in antigen-challenged guinea pig airways.

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Research Article

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Antibody To VLA-4, but Not To L-Selectin, Protects Neuronal M_2 Muscarinic Receptors In Antigen-challenged Guinea Pig Airways

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Abstract

Antigen challenge of sensitized guinea pigs decreases the function of inhibitory M_2 muscarinic autoreceptors on parasympathetic nerves in the lung, potentiating vagally induced bronchoconstriction. Loss of M_2 receptor function is associated with the accumulation of eosinophils around airway nerves. To determine whether recruitment of eosinophils via expression of VLA-4 and L-selectin is critical for loss of M_2 receptor function, guinea pigs were pretreated with monoclonal antibodies to VLA-4 (HP1/2) or L-selectin (LAM1-116). Guinea pigs were sensitized and challenged with ovalbumin, and M_2 receptor function was tested. In controls, blockade of neuronal M_2 muscarinic receptors by gallamine potentiated vagally induced bronchoconstriction, while in challenged animals this effect was markedly reduced, confirming M_2 receptor dysfunction. Pretreatment with HP1/2, but not with LAM1-116, protected M_2 receptor function in the antigen-challenged animals. HP1/2 also inhibited the development of hyperresponsiveness, and selectively inhibited accumulation of eosinophils in the lungs as measured by lavage and histology. Thus, inhibition of eosinophil influx into the lungs protects the function of M_2 muscarinic receptors, and in so doing, prevents hyperresponsiveness in antigen-challenged guinea pigs. (*J. Clin. Invest.* 1997. 99:2036–2044.)

Key words: airway hyperresponsiveness • eosinophils • HP1/2 • hyperreactivity • inflammation

Introduction

Inhalation of aerosolized antigen by sensitized animals or by man causes immediate bronchoconstriction, followed by a period when the lung is hyperresponsive, or abnormally sensitive to a variety of bronchoconstrictive stimuli (1). Antigen-induced hyperresponsiveness in humans and in experimental animals is associated with an influx of inflammatory cells, especially eosinophils, into the airways (2–7). Eosinophils seem to be particularly important for the development of hyperresponsiveness, since pharmacological or immunological depletion or prevention of eosinophil accumulation in the lungs prevents antigen-induced hyperresponsiveness (5, 8–11).

In the lungs, the parasympathetic nerves in the vagi provide the dominant autonomic control of airway smooth muscle (12). These nerves release acetylcholine onto M_3 muscarinic receptors on the smooth muscle, causing contraction of the muscle, and bronchoconstriction (13, 14). Normal control of acetylcholine release from the parasympathetic nerves is provided by inhibitory M_2 muscarinic receptors located on the parasympathetic nerves (15, 16). Stimulating these neuronal M_2 muscarinic receptors with agonists (including acetylcholine) inhibits the release of acetylcholine, and decreases vagally induced bronchoconstriction. Conversely, blocking the neuronal M_2 muscarinic receptors using the selective antagonist gallamine, potentiates vagally induced bronchoconstriction (15). These inhibitory M_2 receptors are important modulators of neurotransmitter release since blocking their function increases acetylcholine release threefold (17), and increases vagally induced bronchoconstriction 5–10-fold (15, 16).

Histological examination of airways from antigen-challenged guinea pigs shows that eosinophils appear to congregate around the nerves, and are occasionally seen within the nerve bundles (18, 19). The presence of eosinophils around the nerves, and thus around the M_2 muscarinic receptors, suggests a mechanism for antigen-induced hyperreactivity. Eosinophils release eosinophil major basic protein and eosinophil peroxidase, both of which are endogenous antagonists of inhibitory neuronal M_2 muscarinic receptors (20). Thus, eosinophil products may increase acetylcholine release from the parasympathetic nerves by acting as endogenous antagonists of the inhibitory M_2 receptors.

The function of the neuronal M_2 muscarinic receptors is markedly impaired after antigen challenge of sensitized animals (16). Loss of function of these inhibitory M_2 receptors is characterized by airway hyperresponsivness to electrical stimulation of the vagus nerves, and by a marked attenuation of the potentiation of vagally induced bronchoconstriction by gallamine. Treatment of antigen-challenged animals with heparin or poly-L-glutamate acutely restores neuronal M_2 muscarinic receptor function, and reduces the hyperresponsiveness (21). Since both of these substances bind to and neutralize major basic protein in vitro (22, 23), this suggests a role for eosinophil proteins in loss of M_2 muscarinic receptor function.

Despite the consistent findings of tissue eosinophilia in allergic diseases of the airways, the mechanisms by which eosinophils are selectively recruited to these tissue sites remains incompletely defined. One possible pathway suggested by recent studies involves differences in adhesion molecules expressed on leukocyte subtypes (24). For example, the β1 integrin very

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late activation antigen-4 (VLA-4)[1] is expressed on eosinophils, but not on neutrophils (25–28). Thus, VLA-4 expression allows eosinophils to adhere to their endothelial counterreceptor, vascular cell adhesion molecule (VCAM-1) (29), while neutrophils cannot. This interaction also enhances activation of eosinophils (30). Endothelial expression of VCAM-1 is increased at sites of tissue eosinophilia, including experimental late-phase reactions of the airways in nasal polyps, and in patients with active asthma (31–36), lending further support to the hypothesis that these adhesion molecules contribute to selective eosinophil recruitment in vivo.

HP1/2 is a mouse monoclonal antibody against human VLA-4 that inhibits eosinophil migration into skin after intradermal challenge of sensitized guinea pigs with antigen (37). In addition, pretreatment with HP1/2 before antigen challenge of sensitized guinea pigs prevents both the accumulation of eosinophils in the lung and hyperresponsiveness normally seen after antigen challenge (11, 37). The experiments reported here were carried out to establish whether pretreatment with an antibody to VLA-4 can also protect the neuronal M_2 muscarinic receptor from antigen challenge, and to determine whether it is specifically eosinophils that are responsible for loss of M_2 muscarinic receptor function and for the development of hyperreactivity in antigen-challenged guinea pigs. In addition, we have compared the effect of the VLA-4 antibody to that of an anti-L-selectin antibody, LAM1-116, because L-selectin is known to function in eosinophil adhesion-related responses in vitro and in vivo (38, 39).

Methods

Animals. Specific pathogen-free Dunkin-Hartley guinea pigs were used (all females, 180–200 g; Hilltop Lab Animals, Inc., Scottsdale, PA). Animals were shipped in filtered crates, and were kept in high-efficiency particulate-filtered (HEPA) air on a normal diet (Prolab; Agway, Syracuse, NY). Guinea pigs 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.

Drugs. Atropine, gallamine, guanethidine, isoproterenol, ovalbumin, suxamethonium, and urethane were purchased from Sigma (St. Louis, MO). All drugs were dissolved and diluted in 0.9% NaCl except the aerosolized ovalbumin, which was dissolved in sterile H_2O.

Monoclonal antibodies. HP1/2, a mouse IgG1 anti-human VLA-4 monoclonal antibody (mAb) that is cross-reactive with guinea pig VLA-4, was generated endotoxin-free for use in these studies as described (37).

LAM1-116, a mouse IgG1 anti-human L-selectin monoclonal was generated by immunizing L-selectin–deficient mice with human L-selectin (40, 41). The LAM1-116 mAb reacts specifically with human, mouse, rat, and guinea pig L-selectin, and does not bind human E or P-selectin. This monoclonal binds to the lectin domain of human L-selectin, and blocks the binding of 95% of human and mouse L-selectin to endothelial venules of peripheral lymph nodes from rat and mouse (Steeber, D.A., and T.F. Tedder, unpublished data). Milligram quantities of endotoxin-free antibodies were purified from sterile mouse ascites fluid by salt extraction followed by anion-exchange chromatography.

Time course of sensitization and challenge with antigen. Guinea pigs were injected intraperitoneally with either saline (control) or 10 $mg \cdot kg^{-1}$ ovalbumin on days 1, 3, and 5 (sensitized) (16). 21 d later animals were antigen-challenged by exposure on four consecutive days to an aerosol of 2.5% ovalbumin for either 5 min, or until signs of respiratory distress appeared. On day 1 of antigen challenge only, pyrilamine (1 $mg \cdot kg^{-1}$) was administered intraperitoneally to all animals 60 min before exposure to aerosolized ovalbumin. 24 h after the last antigen challenge, the function of the neuronal M_2 muscarinic receptors, and the inflammatory cells in blood, in bronchoalveolar lavage, and in airway tissue, were measured.

Two groups of guinea pigs were sensitized with ovalbumin as above, but were treated with either HP 1/2 (4 $mg \cdot kg^{-1}$ ip) or LAM1-116 (4 $mg \cdot kg^{-1}$ ip) 1 h before challenge. Animals received four separate injections, one per day on each of the four challenge days. These doses were selected based on results from pilot studies in four animals with HP1/2, demonstrating that at the end of this protocol serum levels of mouse monoclonal antibodies, as determined by ELISA, were sufficient to saturate surface ligands (33 ± 10 μg/ml; see below).

Anesthesia. Guinea pigs were anesthetized with 1.5 $g \cdot kg^{-1}$ urethane intraperitoneally. While this dose of urethane produced a deep anesthesia lasting 8–10 h, none of the experiments lasted for longer than 3 h (42).

A carotid artery was cannulated for measurement of blood pressure using a transducer (Spectramed, Oxnard, CA), and the heart rate was derived from the blood pressure using a tachograph. Both vagus nerves were cut, and the distal ends were placed on electrodes and bathed in a pool of liquid paraffin. The animals were paralyzed by infusing suxamethonium (10 $\mu g \cdot kg^{-1}\ min^{-1}$). A positive pressure, constant volume rodent ventilator (Harvard Apparatus, Inc., South Natick, MA) was used to artificially ventilate the animals via a tracheal cannula at a tidal volume of 1.0 ml/100 g body wt and 100 breaths·min^{-1}. Pulmonary inflation pressure (Ppi) was measured at the trachea using a pressure transducer (DTX, Spectramed). A positive pressure of 80–150 mm H_2O was needed to adequately ventilate the animals. Signals were recorded on a polygraph (Grass Instrument Co., Quincy, MA). Bronchoconstriction was measured as the increase in Ppi over the basal inflation pressure produced by the ventilator (43). The sensitivity of the method was increased by taking the output Ppi signal from the driver of one channel to the input of the preamplifier of a different channel on the polygraph. Thus, baseline Ppi was recorded on one channel, and increases in Ppi above the baseline were recorded on a separate channel. This method provided a way to balance the baseline Ppi signal on the latter channel, and to record the increase in Ppi at a greater amplification. With this method, increases in Ppi as small as 2–3 mm H_2O could be accurately recorded.

Measurement of hyperreactivity. All animals were pretreated with guanethidine (5 $mg \cdot kg^{-1}$ ip) to deplete noradrenaline (44). Electrical stimulation of both vagus nerves produced bronchoconstriction (measured as an increase in Ppi) and bradycardia. Pulse duration (0.2 ms), the volts used (10 V), and the train stimulus (5 s) were kept constant, while the frequency was increased from 2–15 Hz. Supramaximal voltages were not used since increasing the volts above 10 caused severe bradycardia. Likewise, frequencies above 15 Hz were also not used because of the severe bradycardia. The vagus nerves were stimulated at 2-min intervals. Vagally induced bronchoconstriction was measured as the peak increase in Ppi above basal inflation pressure in mm H_2O.

Measurement of M_2 muscarinic receptor function. Electrical stimulation of both vagus nerves produced bronchoconstriction (measured as an increase in Ppi) and bradycardia. The nerves were stimulated at 1-min intervals. The frequency (15 Hz), pulse duration (0.2 ms), and train stimulus (5 s) were kept constant for each group of animals. All animals were pretreated with guanethidine (5 $mg \cdot kg^{-1}$ ip) to deplete noradrenaline (44). Before giving gallamine, the voltage was chosen to give a bronchoconstriction of 15–20 mm H_2O (9.5 ± 0.78 V). Cumulative doses of gallamine (0.1–10.0 $mg \cdot kg^{-1}$) were administered, and the effects of gallamine on vagally induced bronchoconstriction were measured as a ratio of bronchoconstriction in the presence of gallamine to the bronchoconstriction in the absence of gallamine.

1. *Abbreviations used in this paper:* Ppi, pulmonary inflation pressure; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.

At the end of each experiment, atropine (1 mg·kg^{-1} iv) blocked all responses to vagal nerve stimulation, demonstrating that bronchoconstriction was mediated via muscarinic receptors. A few sensitized guinea pigs were randomly selected and given ovalbumin (1 mg·kg^{-1} iv) to demonstrate that the sensitized animals were indeed sensitized.

Bronchoalveolar lavage and peripheral blood analysis. After the function of the neuronal M_2 muscarinic receptor had been measured using gallamine, bronchoalveolar lavage (BAL) was performed in situ via the tracheal cannula. The lungs were lavaged with 5 aliquots of 10 ml warm PBS containing 3 mM EDTA and 100 μM isoproterenol (pH 7.2–7.4) (45). The recovered lavage fluid (40–45 ml) was centrifuged, the cells were resuspended in 20 ml of PBS, and total cells were counted using a hemacytometer. Aliquots of the cell suspension were cytospun onto glass slides, stained, (Diff-Quik®; Scientific Products, McGaw Park, IL) and counted to obtain cell differentials (46). Whole blood was taken from the carotid cannula via a heparinized syringe, and was placed in 0.1 N HCl solution to lyse red blood cells. Total peripheral blood leukocytes were counted with a hemacytometer, and cell differentials were obtained from a blood smear.

Flow cytometry. 100-μl aliquots of EDTA-anticoagulated guinea pig blood from untreated animals were incubated with saturating concentrations (5 μg/ml) of HP1/2, LAM1-116, or with identical concentrations of an irrelevant mouse IgG1 monoclonal (Coulter Immunology, Hialeah, FL) essentially as described (47). After erythrocyte lysis, cells were incubated with saturating concentrations of FITC-conjugated polyclonal goat anti-mouse IgG antiserum (Caltag Laboratories, South San Francisco, CA), fixed, and neutrophils and lymphocytes were analyzed by appropriate gating for immunofluorescence using a flow cytometer (EPICS; Coulter) after excitation at 488 nm. At least 5,000 events were collected. For comparison in experiments where animals received antibody treatments in vivo, leukocytes were obtained at the end of the experiments, and attachment of monoclonal antibodies was detected by the addition of the FITC-conjugated secondary antibody alone; controls consisted of no secondary antibody (see Fig. 1). To further investigate whether antibody levels were saturating and were maintained in vivo, separate aliquots of cells from antibody-treated animals were preincubated in vitro with saturating concentrations of monoclonal antibody, and were processed in the same manner as the cells from the nonantibody-treated animals. This approach confirmed that saturating levels of antibody were already present on the circulating cells (i.e., no increase in labeling was observed), and that antibody treatment in vivo did not result in any detectable changes in expression of VLA-4 (on lymphocytes) or L-selectin (on lymphocytes or neutrophils) (data not shown).

Pathological evaluation. The guinea pig lungs were perfused and fixed using an adaptation of the method described by Bassett and Fisher (48). In brief, the whole animal was exsanguinated by perfusing the hepatic portal vein with warm saline and by cutting the dorsal aorta. The lungs were then directly perfused with warm 0.9% saline via the pulmonary artery until they were white. The lungs were fixed by way of the pulmonary artery using warmed fixative (1% glutaraldehyde, 1% paraformaldehyde, and 1% sucrose in 0.1 M cacodylate buffer; pH 7.2–7.4). The fixation procedure lasted for 15 min at a rate of 10–12 ml·min^{-1} (Masterflex pump system; Cole-Palmer Instrument Co., Chicago, IL) with the lung volume set at functional residual capacity. Transverse sections from the trachea and from each lobe of the lung (3–5 mm thickness) were taken for pathological evaluation. Sections were embedded in glycol methacrylate for light microscopy (JB-4; Polysciences, Inc., Warrington, PA) (49).

Statistics. Frequency response curves in control and antigen-challenged guinea pigs were compared using a two-way analysis of variance for repeated measures. Dose–response curves to gallamine in control and antigen-challenged guinea pigs were also compared using a two-way analysis of variance for repeated measures. The initial bronchoconstrictor responses to stimulation of the vagus nerves between control and antigen-challenged guinea pigs were compared by using unpaired Student's *t* tests (50). The differences in cells recovered from either BAL or peripheral blood between treatment groups were tested by use of a one-factor analysis of variance (50). $P < 0.05$ was considered significant.

Results

Several of the sensitized nonantibody-treated animals were injected with intravenous ovalbumin after measuring the function of the neuronal M_2 muscarinic receptors. This injection caused a sustained bronchoconstriction (in excess of 500 mm H_2O), confirming that these animals were sensitized to ovalbumin (antibody-treated guinea pigs were not tested in this manner since the resulting bronchoconstriction makes lavage impossible.) In the antibody-treated guinea pigs, leukocytes were obtained at the end of the experiments, and saturation with antibody was confirmed by flow cytometry (Fig. 1).

In the absence of electrical stimulation of the vagus nerves, baseline Ppi was not significantly different among control animals (113.1±5.1 mm H_2O), animals that were sensitized and challenged with ovalbumin (126.6±4.5 mm H_2O), challenged animals pretreated with HP1/2 (140.5±14.4 mm H_2O), or challenged animals pretreated with LAM1-116 (105.4±8.6 mm H_2O). Likewise, there was no significant difference in baseline heart rate among control animals (294.3±6.2 beats·min^{-1}), animals that were sensitized and challenged with ovalbumin (301.4±8.9 beats·min^{-1}), challenged animals pretreated with HP1/2 (279.3±7.8 beats·min^{-1}), or challenged animals pretreated with LAM1-116 (307.5±11.1 beats·min^{-1}).

Simultaneous electrical stimulation of both vagus nerves resulted in bronchoconstriction, which was rapidly reversed after electrical stimulation of the nerves was stopped. Electrical stimulation of the nerves (10 V, 0.2 ms, 5-s duration, 2–15 Hz) produced a frequency-dependent increase in vagally induced bronchoconstriction in control animals (Fig. 2, *open squares*). This increase was significantly greater in animals that had been sensitized and challenged (Fig. 2, *closed squares*). Pretreatment with HP1/2 prevented the increased response to electrical stimulation of the nerves (Fig. 2, *closed circles*).

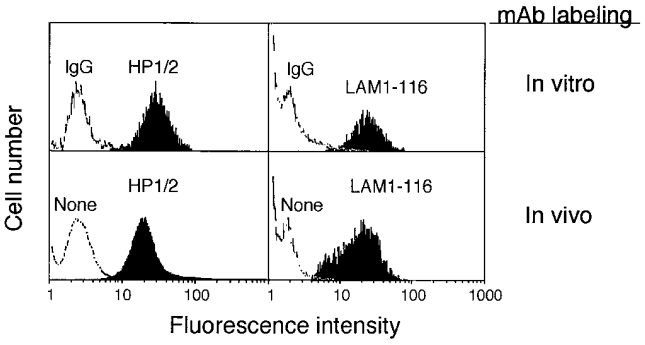

Figure 1. Flow cytometric analysis of VLA-4 and L-selectin expression on guinea pig peripheral blood lymphocytes. Blood was obtained 24 h after daily administration of 4 mg·kg^{-1} of either HP1/2 or LAM1-116 for four consecutive days, and leukocytes were labeled by incubation in vitro with FITC anti-mouse IgG (displayed as in vivo). For comparison, blood was simultaneously obtained from a nonantibody-treated animal, and was labeled in vitro with HP1/2 or LAM1-116, followed by incubation in vitro with FITC anti-mouse IgG (displayed as in vitro). Lymphocytes were identified based on their typical light scatter characteristics. Data are representative of three separate experiments yielding similar results.

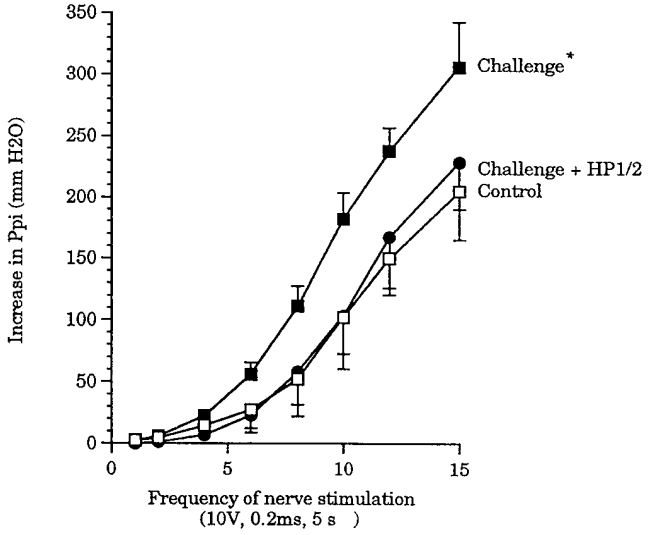

Figure 2. The potentiation of vagally induced bronchoconstriction after antigen challenge is inhibited by pretreatment with the mAb HP1/2. In control animals (*open squares*), electrical stimulation of the vagus nerves (10 V, 0.2 ms, 5 s) at increasing frequencies (2–15 Hz) causes frequency-dependent bronchoconstriction, measured as an increase in pulmonary inflation pressure in mm H_2O. Frequency-dependent bronchoconstriction was significantly greater in animals that had been antigen-challenged (*closed squares*), but was not different from control in animals pretreated with the mAb HP1/2 before antigen challenge (*closed circles*). Each point is the mean±SEM; $n = 5$ animals. *Significantly different from control.

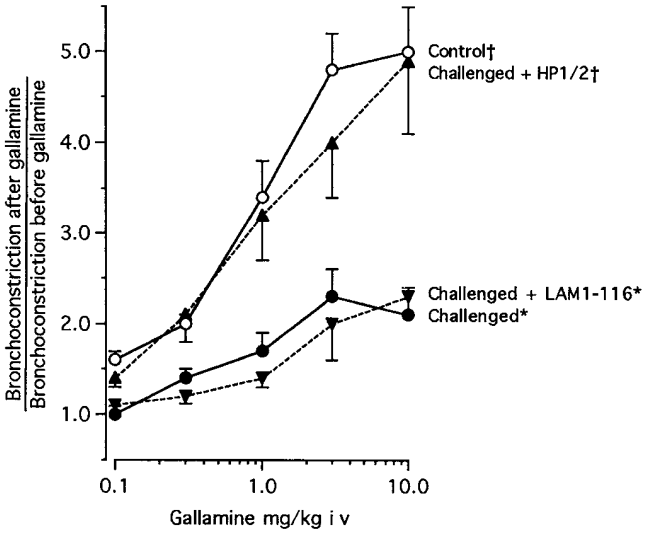

Figure 3. The potentiation of vagally-induced bronchoconstriction by gallamine is protected by pretreatment with HP1/2 in antigen-challenged animals. In the absence of gallamine, electrical stimulation of the vagus nerves (15 Hz, 0.2 ms, 9.5±0.78 V, 75 pulses/train) causes bronchoconstriction measured as a rise in pulmonary inflation pressure in mm H_2O. There was no significant difference between these groups. Gallamine potentiates vagally induced bronchoconstriction in control animals (*open circles*), but not in antigen-challenged animals (*solid circles*; $P < 0.0001$). In antigen-challenged guinea pigs pretreated with HP1/2, gallamine potentiated vagally-induced bronchoconstriction (*upturned solid triangles*). There was no significant difference between control guinea pigs and antigen-challenged guinea pigs that were pretreated with HP1/2, but there was a difference between the HP1/2-treated and untreated antigen-challenged groups; $P < 0.0006$). Pretreatment with LAM1-116 did not significantly affect the response of the antigen-challenged animals to gallamine. The results are expressed as a ratio of vagally induced bronchoconstriction in the presence of gallamine to vagally induced bronchoconstriction in the absence of gallamine. Each point is the mean±SEM; $n = 5$ animals. *Significantly different from control; †significantly different from antigen-challenged.

In the absence of gallamine, electrical stimulation of both vagus nerves (15 Hz, 0.2 ms for 5 s; at 1-min intervals) was slightly adjusted (5–20 V), to induce bronchoconstrictions that were similar among groups (19.0±0.9 mm H_2O in controls; 19.4±1.0 mm H_2O in antigen-challenged guinea pigs; 17.7±1.0 mm H_2O in antigen-challenged guinea pigs pretreated with HP1/2, and 16.2±0.6 mm H_2O in antigen-challenged guinea pigs pretreated with LAM1-116).

In control animals, gallamine (0.1–10 mg·kg^{-1} iv) potentiated vagally-induced bronchoconstriction in a dose-dependent fashion by blocking inhibitory M_2 muscarinic receptors on the pulmonary parasympathetic nerves (Fig. 3, *open circles*). The effect of gallamine was quite marked; 10 mg·kg^{-1} increased vagally induced bronchoconstriction fivefold. In contrast, potentiation of vagally induced bronchoconstriction by gallamine was significantly attenuated in sensitized, challenged animals (Fig. 3, *solid circles*). In those animals pretreated with HP1/2 before antigen challenge, the response to gallamine was not significantly different from the controls (Fig. 3, *solid triangles*). Pretreatment with LAM1-116 before antigen challenge did not affect the response of the antigen-challenged animals to gallamine (Fig. 3, *solid, upside-down triangles*).

In the heart, gallamine caused a dose-related inhibition of vagally induced bradycardia. The effect of gallamine on vagally induced bradycardia was not different among control, sensitized, and challenged or challenged animals pretreated with HP1/2 or LAM1-116 (data not shown).

Antigen challenge significantly increased the total number of inflammatory cells recovered from the lungs by bronchoalveolar lavage (the volume recovered was not different between groups). This increase was comprised primarily of eosinophils (Fig. 4, compare solid to open bars). Pretreatment with HP1/2 before antigen challenge inhibited this increase in eosinophils without affecting the number of macrophages, neutrophils, or lymphocytes in the lavage fluid (Fig. 4, compare hatched bars to solid bars). Pretreatment with LAM1-116 before antigen challenge did not significantly inhibit the influx of eosinophils (Fig. 4).

Histological examination of airways demonstrated that eosinophils were also increased in the lungs of antigen-challenged guinea pigs. There were few inflammatory cells in the airways of control guinea pigs. Similarly, there were no eosinophils around the nerve bundles below the airway smooth muscle (Fig. 5). In contrast, in antigen-challenged guinea pigs, the airways, particularly the area around the nerves, were filled with inflammatory cells, primarily eosinophils (Fig. 6). Eosinophilic inflammation of the nerve bundles was blocked in the guinea pigs pretreated with HP1/2 (Fig. 7), but was not blocked by pretreatment with LAM1-116 (Fig. 8).

Antigen challenge in the absence or presence of LAM1-116 did not affect the number or types of circulating leukocytes

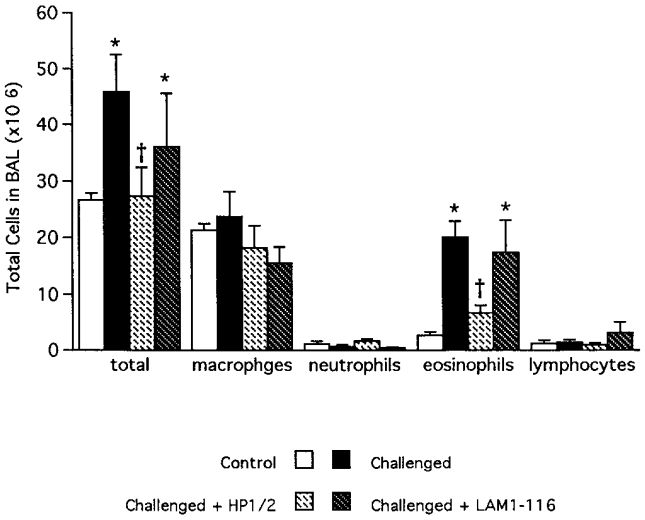

Figure 4. Treatment with HP1/2 inhibited the influx of eosinophils into the lungs following antigen challenge. The total number of inflammatory cells obtained from bronchoalveolar lavage (BAL) was significantly increased in the challenged guinea pigs (*solid bars*) compared with the controls (*open bars*). This increase was predominantly eosinophils. Treatment with HP1/2 (*dashed bars*) prevented the increase in total inflammatory cells and in eosinophils. Treatment with LAM1-116 (*striped bar*) did not significantly affect either the increase in total cells or the increase in eosinophils. Data are expressed as the mean±SEM, $n = 5$. *Significantly different from control; †significantly different from antigen-challenged.

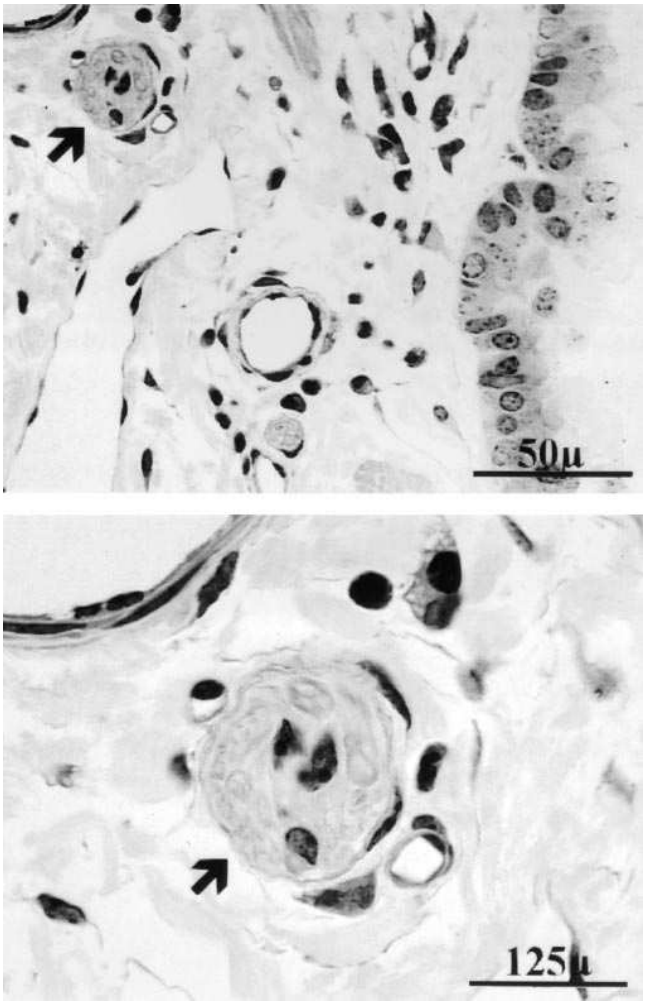

Figure 5. There are few inflammatory cells and no eosinophils around the nerves in lungs from control guinea pigs. Sections of lung were embedded in methacrylate, sectioned, and stained with hematoxylin and eosin. A nerve bundle (*arrow*) is shown lying under the airway smooth muscle under low (*top*) and high magnification (*bottom*).

measured 24 h after the fourth antigen challenge (which coincides with 24 h after the fourth mAb dose). Pretreatment with HP1/2, however, significantly increased the number of circulating leukocytes at this same time point. This increase appeared to be made up predominantly of lymphocytes (Fig. 9).

Discussion

In the lungs, neuronal M_2 muscarinic receptors normally function to inhibit release of acetylcholine from the parasympathetic nerves. These receptors are important neuromodulators of nerve function, since in control (unchallenged) guinea pigs, blockade by gallamine potentiated vagally induced bronchoconstriction fivefold. These data confirm previous findings (15). In contrast, the gallamine-induced potentiation of vagally induced bronchoconstriction was markedly attenuated after antigen challenge. These results confirm that the function of neuronal M_2 receptors in the lung is impaired after antigen challenge (16, 21).

Since neuronal M_2 muscarinic receptors inhibit acetylcholine release, loss of M_2 receptor function is a potential mechanism for antigen-induced hyperresponsiveness. Frequency response curves were significantly potentiated after antigen challenge (Fig. 2), indicating that the animals were hyperresponsive. Pretreatment of sensitized guinea pigs with the mAb HP1/2, but not with the mAb LAM1-116, prevented loss of M_2 muscarinic receptor function in antigen-challenged animals (Fig. 3). In addition, pretreatment with the mAb HP1/2 also prevented the increase in hyperreactivity after antigen challenge (Fig. 2). These data strongly suggest that the hyperresponsiveness to nerve stimulation after antigen challenge is mediated by loss of neuronal M_2 muscarinic receptor function.

The effects of HP1/2 on the function of the neuronal M_2 receptors could not be accounted for by changes in baseline resistance, since baseline Ppi was the same in control and in antigen-challenged guinea pigs in the absence and presence of HP1/2 (or LAM1-116). Circulating inflammatory cells were not altered by antigen challenge, or by treatment with any of the antibodies (with the exception of lymphocytes, which were increased following treatment with HP1/2) (Fig. 9).

The increase in circulating lymphocytes after HP1/2 is a consistent finding in many animal models (51). The mechanism responsible for this increase is unknown. While it was initially assumed that these effects were due to interference with normal lymphocyte recirculation patterns to mesenteric sites via blockade of $\alpha 4\ \beta 7$ function, this assumption does not appear to be the case (52). Although inhibition of lymphocyte recruitment to the airways has been seen with $\alpha 4$ integrin antibody treatment in a mouse model of allergic airways disease

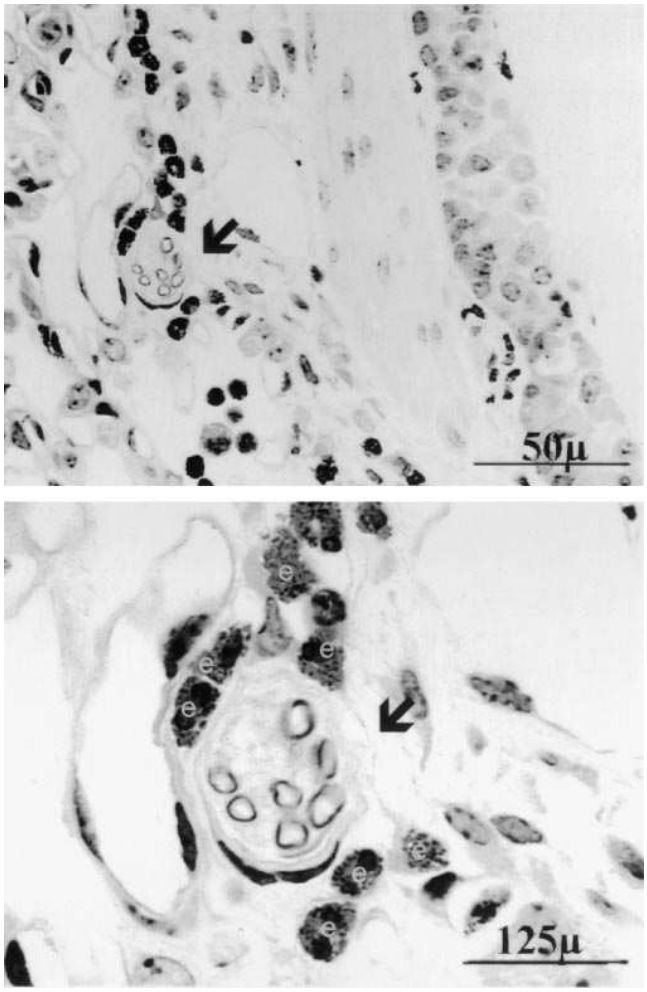

Figure 6. Inflammatory cells are present in the lungs of guinea pigs that have been sensitized and challenged with antigen. Lung tissue was processed as in Fig. 5. A nerve bundle (*arrow*) is shown lying under the airway smooth muscle under low (*top*) and high magnification (*bottom*). In these antigen-challenged guinea pigs, eosinophils (*e*) surround the nerves.

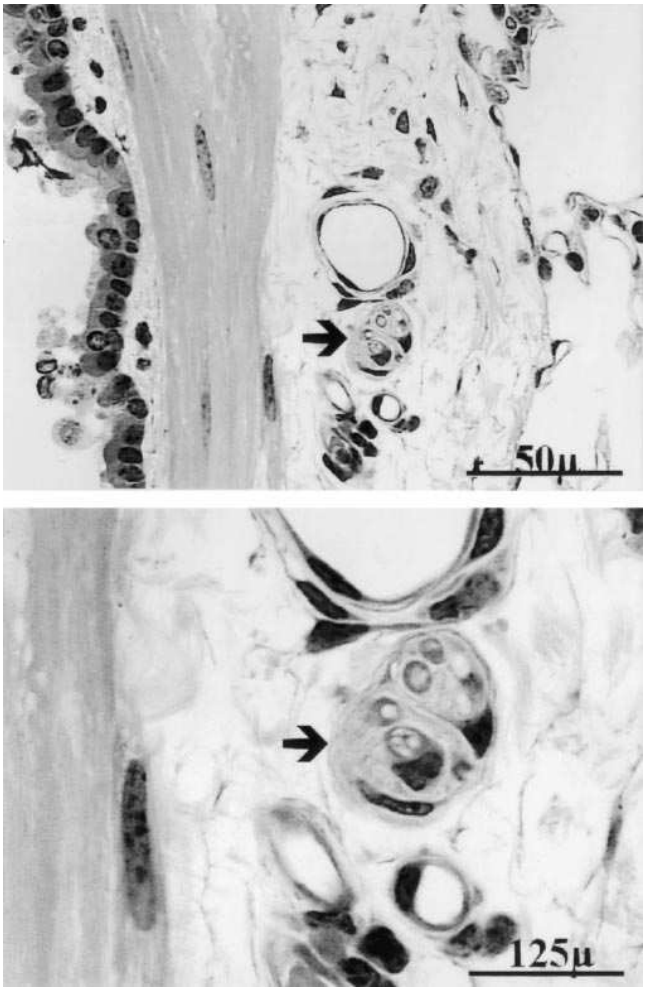

Figure 7. The lungs of guinea pigs that have been treated with HP1/2 before antigen challenge have a marked absence of eosinophils around their nerves. Lung tissue was processed as in Fig. 5. A nerve bundle (*arrow*) is shown lying under the airway smooth muscle under low (*top*) and high magnification (*bottom*).

(9), this inhibition was not observed in our study. Therefore, it seems unlikely that the lymphocytosis seen in the present experiments was due to the inhibition of lymphocyte migration into the airways.

Antigen challenge is associated with inflammation, particularly by eosinophils, of the airways and lungs (5, 8–11). Bronchoalveolar lavage of the airways showed a selective and significant increase in the numbers of eosinophils in the challenged guinea pigs when compared with the controls. This increase was inhibited by pretreatment with the antibody HP1/2, but not by pretreatment with the antibody LAM1-116 (Fig. 3). In addition, histological examination of the airways of challenged guinea pigs showed an accumulation of eosinophils around the nerves of the challenged animals that was inhibited by pretreatment with HP1/2, but not by LAM1-116. Thus, pretreatment with a VLA-4 mAb selectively inhibited eosinophil migration into the airways of antigen-challenged guinea pigs, and this inhibition was associated with the protected function of the neuronal M_2 muscarinic receptors. Conversely, the antibody to L-selectin, LAM1-116, had no effect on either eosinophil migration, or on M_2 muscarinic receptor function.

Other studies have demonstrated that inhibition of eosinophil influx into the airways prevents antigen-induced hyperresponsiveness (11, 53). We have confirmed these studies by demonstrating that the mAb HP1/2 also prevents antigen-induced hyperresponsiveness. Furthermore, we have demonstrated that inhibition of eosinophil influx into the airways prevents antigen-induced loss of M_2 muscarinic receptor function. Since loss of receptor function causes hyperresponsiveness of the vagus nerves (15–17), and thus hyperresponsiveness to any challenge that has a vagal component, eosinophils may induce hyperreactivity by interfering with neuronal M_2 muscarinic receptor function.

The inability of the L-selectin mAb LAM1-116 to prevent eosinophil recruitment into the lung, and to prevent allergen-induced changes in M_2 muscarinic receptor function, strongly suggests that L-selectin function is not critical for eosinophil migration into the lung in this model. L-selectin blockade with

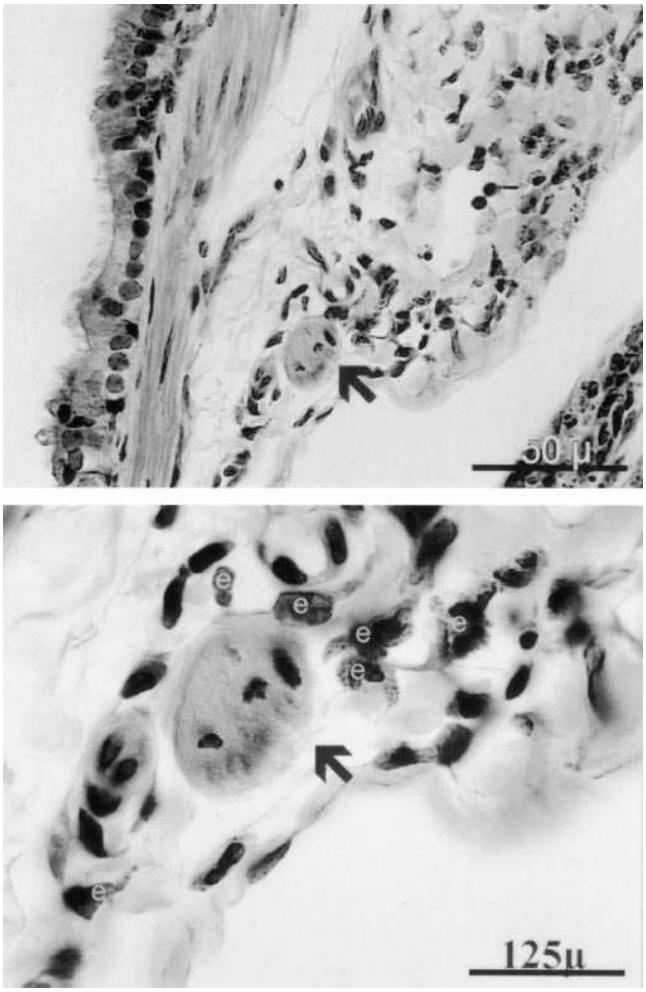

Figure 8. The lungs of guinea pigs that have been treated with LAM1-116 before antigen challenge still demonstrate eosinophils (*e*) around their nerves. Lung tissue was processed as in Fig. 5. A nerve bundle (*arrow*) is shown lying under the airway smooth muscle under low (*top*) and high magnification (*bottom*).

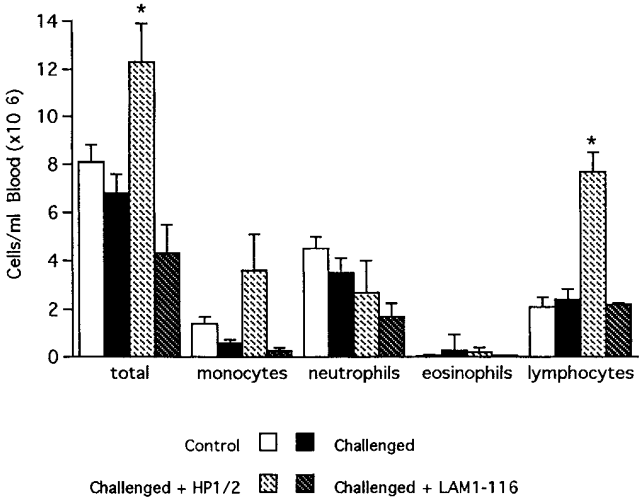

Figure 9. In the peripheral blood, there was no statistical difference in circulating leukocyte numbers 24 h after the fourth day of experimentation among the control (*open bars*), challenged (*solid bars*), or LAM1-116 treated and challenged (*striped bars*) groups. Pretreatment with HP1/2 (*dashed bars*), however, increased the total number of circulating lymphocytes in the challenged animals. Data are expressed as the mean±SEM, $n = 5$. * Significantly different from control.

mAb both in vitro and in vivo has been shown to reduce eosinophil rolling responses (38, 39). In other animal models, attenuation of L-selectin function or expression clearly reduces neutrophil recruitment (41, 54–57). The inability of LAM1-116 to block eosinophil recruitment was not due to inadequate dosing, because maintenance of saturating serum levels was verified by flow cytometry (Fig. 1). Furthermore, the LAM1-116 mAb, when administered at similar doses, has been used successfully in mouse models to prevent leukocyte influx into sites of inflammation (Steeber, D.A., and T.F. Tedder, unpublished data). Thus, it may be possible that L-selectin is more important in neutrophil recruitment responses than for eosinophil recruitment.

Since LAM1-116 had no effect on eosinophil influx in antigen-challenged guinea pigs, any possible concerns that irrelevant, nonbinding isotype-matched mAb (used as protein controls in previous studies) might have yielded different results, can be eliminated.

Since eosinophils surround the nerves of antigen-challenged guinea pigs, we have previously suggested that eosinophils play an important role in antigen-induced loss of M_2 muscarinic receptor function (19). Eosinophil major basic protein has been identified immunohistologically in the nerve bundles of antigen-challenged guinea pigs, and in humans with asthma (18, 58). Both eosinophil major basic protein and eosinophil peroxidase are allosteric antagonists of the M_2 muscarinic receptor (20). An association between eosinophil products and loss of M_2 muscarinic receptor function in vivo was first suggested by the demonstration that polyanionic substances such as heparin, that bind and neutralize eosinophil major basic protein in vitro, acutely restore M_2 receptor function in vivo (21). Thus, eosinophil major basic protein and eosinophil peroxidase may act as endogenous inhibitors of M_2 receptor function. Selective depletion of eosinophils using a mAb against interleukin-5 also protects neuronal M_2 muscarinic receptors, supporting a role for eosinophils in loss of receptor function (19).

The mechanisms of eosinophil recruitment to the nerves in the lungs are unknown. There is now extensive evidence in the literature that the receptor for VLA-4, VCAM-1, is expressed at sites of allergic inflammation in vivo. Allergen challenge of allergic individuals activates the vascular endothelium to express adhesion molecules including VCAM-1 (32, 59, 60); a significant correlation between VCAM-1 staining and eosinophil influx is often seen. There is also indirect evidence that VCAM-1 expression occurs within the human airway after endoscopic intrabronchial antigen challenge, since increased levels of a soluble form of VCAM-1 has been detected in BAL fluids (61). Additional experiments implicate VCAM-1 in the pathophysiology of allergic rhinitis and asthma in humans. In one such study, strong endothelial staining for VCAM-1, as well as for ICAM-1, was observed in endobronchial biopsy specimens from symptomatic patients with asthma (33). Studies of nasal airway tissue from subjects with perennial allergic rhinitis have modestly increased expression of VCAM-1 com-

pared with tissues from nonallergic controls, and significant VCAM-1 staining has been reported in nasal polyps, tissues extensively infiltrated by eosinophils (31, 34).

In the nerves, VCAM-1 can be expressed after incubation with tumor necrosis factor (62). Thus, it is possible that cytokine-induced VCAM-1 expression might play a role in the observed association between eosinophils and airway nerves. It is also tempting to speculate that antigen challenge induces VCAM-1 expression in the airways, and may also induce expression by the nerves in the airway.

Alternatively, another ligand for VLA-4 is a form of alternatively spliced fibronectin containing CS-1 (63). While VLA-4 binds less well to fibronectin than to VCAM-1, fibronectin is expressed by the microvasculature (64, 65), and it is possible that it may be involved in localizing eosinophils within the lung.

Once the eosinophils have migrated out of the vasculature, many localize to the nerves within the airways (18, 58). These eosinophils subsequently release granule proteins, including eosinophil major basic protein and eosinophil peroxidase, which by inhibiting neuronal M_2 muscarinic receptors, increase release of acetylcholine from the vagus nerves, resulting in antigen-induced hyperresponsiveness. Blockade of VLA-4 with the mAb HP1/2 inhibits migration of eosinophils, protecting the neuronal M_2 muscarinic receptors, and preventing antigen-induced hyperresponsiveness.

Although antibody-blocking data for allergic diseases in humans is lacking, blocking mAbs to VLA-4 have been infused in vivo in a variety of animal models of eosinophilic allergic inflammatory conditions of the lung (9, 11, 37, 53, 66–68). In general, these studies agree that pretreatment with VLA-4 mAb will reduce or prevent eosinophil influx into the lungs, and prevent the physiological changes that accompany this reaction. An example of an exception to this rule was seen in the sheep model, where infusion of a VLA-4 antibody prevented late-phase changes in airway function without affecting eosinophil recruitment as assessed by BAL (53).

In conclusion, the data presented here delineate the role of VLA-4, and not L-selectin, in allergen-induced eosinophil recruitment to the guinea pig airway, in the localization of recruited eosinophils to nerves, and in the alteration in neuronal M_2 muscarinic receptor function and subsequent hyperresponsiveness. While other mediators, such as eotaxin and other C-C chemokines (69–71), may also be important in eosinophil recruitment and localization in the airways, eosinophil VLA-4, presumably via its interactions with counterligands VCAM-1 (and perhaps fibronectin) appears to be critical in the recruitment response, and lends support to the concept that VLA-4 antagonists may prove useful in the treatment of allergic diseases (51).

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