

## In Vivo Neutralization of Eosinophil-derived Major Basic Protein Inhibits Antigen-induced Bronchial Hyperreactivity in Sensitized Guinea Pigs

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### Abstract

This study examines the effect of purified rabbit anti-guinea pig eosinophil-derived major basic protein (MBP) Ig on antigen-induced bronchial hyperreactivity to inhaled acetylcholine in aerosol-sensitized guinea pigs. Ovalbumin inhalation by sensitized guinea pigs induced a rise in the numbers of eosinophils and in the levels of MBP in the bronchoalveolar lavage fluid, which peaked at 24 h and resolved at 72 h. Antigen-challenged animals exhibited bronchial hyperreactivity to inhaled acetylcholine at 72 h, but not at 6 or 24 h. The intranasal administration of 200  $\mu$ l of purified rabbit anti-guinea pig MBP Ig, at 2.5 mg/ml, but not of the control preimmune rabbit Ig, 1 h before and 5 h after ovalbumin inhalation suppressed bronchial hyperreactivity to acetylcholine at 72 h without affecting the number of eosinophils accumulating in the bronchoalveolar lavage fluid. These findings indicate that antigen challenge in sensitized guinea pigs is followed by early eosinophil infiltration and activation within the airways and by late bronchial hyperreactivity. Neutralization of endogenously secreted MBP by a specific antiserum prevented antigen-induced bronchial hyperreactivity, suggesting that eosinophil degranulation plays an important role in the alterations of bronchopulmonary function in the guinea pig. (*J. Clin. Invest.* 1996. 97:1117–1121.) Key words: bronchoalveolar lavage • eosinophil activation • cationic proteins • enzyme-linked immunosorbent assay

### Introduction

Bronchial hyperreactivity, both in animal models and in humans, is frequently associated with airway inflammation, char-

acterized by eosinophil and mononuclear cell infiltrate in bronchial tissue and bronchoalveolar lavage (BAL)<sup>1</sup> fluid (1, 2). The observation that some of the infiltrating eosinophils appear degranulated (3) suggested a role for their secretory products in the acquisition of tissue injury and the accompanying alterations in lung function (4).

Major basic protein (MBP) is a cationic protein stored in the core of the large crystalloid-containing granules of the eosinophil (5). In vitro incubation of human or guinea pig respiratory epithelium with purified MBP leads to a loss of its integrity (6–8), probably by a mechanism dependent on the high charge of this cationic protein (9). More recently, the in vivo administration of MBP was shown to induce bronchial hyperreactivity in different species (9–12), suggesting a causal association between eosinophil activation and modifications in bronchopulmonary function. This hypothesis was also corroborated by studies demonstrating a significant correlation between the intensity of bronchial hyperreactivity and the levels of eosinophil-derived cationic proteins in blood and BAL fluid from asthmatics (13, 14).

We have shown that ovalbumin-sensitized guinea pigs exposed to a single antigen challenge developed a marked BAL eosinophilia, unaccompanied by changes in airway reactivity to methacholine and in the levels of eosinophil-derived MBP in the BAL fluid (15). However, when eosinophil degranulation was induced by the intratracheal administration of leukotriene B<sub>4</sub>, the airway sensitivity to methacholine was increased and a rise in the concentration of MBP in the supernatant of the BAL fluid was observed. We thus suggested that eosinophil activation, rather than eosinophil accumulation by itself, is required for the onset of bronchial hyperreactivity (15).

To establish more precisely the role of MBP in the development of antigen-induced bronchial hyperreactivity, Igs obtained from rabbits sensitized against purified guinea pig MBP were administered into the airways of aerosol-sensitized animals before and after antigen inhalation. Using this approach, we show that bronchial hyperreactivity to aerosolized acetylcholine, which follows eosinophil accumulation and activation in the BAL fluid in response to antigen challenge, is suppressed by the in vivo neutralization of secreted MBP. Our results suggest that endogenously released MBP by activated

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1. Abbreviations used in this paper: BAL, bronchoalveolar lavage; C<sub>dyn</sub>, dynamic compliance; MBP, major basic protein; PD, provocative dose; R<sub>L</sub>, lung resistance.

eosinophils is involved in the development of bronchial hyperreactivity in this model.

## Methods

**Guinea pig sensitization.** Male Hartley guinea pigs (400–600 g; C. Lebeau, Gambais, France) were immunized by aerosolized ovalbumin (Miles Laboratories, Inc., Naperville, IL), at 1% in sterile 0.9% NaCl (saline) for 30 min, twice at a 2-d interval. 14–17 d after the first inhalation, guinea pigs were exposed for 15 min to five successive solutions of ovalbumin of 0.001, 0.01, 0.1, 0.5, and 1% (16). Control animals were sensitized against ovalbumin and exposed to aerosolized sterile saline for 30 min. The aerosol was delivered into a 36-liter plexiglass chamber using an ultrasonic nebulizer (Ultra-Neb 99; DeVilbiss Medical, Arcueil, France), which produced particles of a mass diameter averaging between 0.5 and 3  $\mu\text{m}$ . The liquid output of the nebulizer was 0.6 ml/min. The animals (6–8 for each group) were used 6, 24, or 72 h after challenge for eosinophil and MBP determinations in the BAL fluid and for measurement of bronchial hyperreactivity.

**Anti-guinea pig MBP Ig preparation and treatments.** Female HY/CR rabbits (2,500 g; Charles River, St. Aubin les Elbœuf, France) were immunized at 2-wk intervals with 50  $\mu\text{g}$  of reduced and alkylated purified guinea pig MBP (a gift from Dr. M.K. Church, Southampton General Hospital, Southampton, UK), emulsified in adjuvant (Hunter TiterMax; CytRx Co., Norcross, GE). The animals were bled 7 d after the second injection, and Ig was obtained after precipitation with 40% saturation of ammonium sulfate. Purified anti-MBP Ig (2.5 mg/ml) was administered into the nostrils of each sensitized guinea pig in a volume of 200  $\mu\text{l}$ , 1 h before and 5 h after antigen challenge. Control animals were treated twice with the same amounts of preimmune Ig. Four to eight animals for each group were used.

**Assessment of bronchial hyperreactivity.** The day of the experiment, i.e., 6, 24, or 72 h after the challenge, guinea pigs were anesthetized by an intraperitoneal injection of 30 mg/kg sodium pentobarbital (Clin-Midy, Montpellier, France), and tracheae were cannulated. Animals were ventilated with a small respiratory pump (Biosciences, Sheerness, UK) at 60 strokes/min and 10 ml/kg body weight. The jugular vein was cannulated, and spontaneous breathing was suppressed by the intravenous injection of 4 mg/kg of pancuronium bromide (Organon Teknika, Fresnes, France). Airflow and transpulmonary pressure were measured and the lung resistance ( $R_L$ ) and dynamic compliance ( $C_{\text{dyn}}$ ) were calculated using a computerized pulmonary monitoring system ( $\mu\text{Med PMS}$ , London, UK).

At least 1 h after surgery, bronchial reactivity was tested by the aerosol administration of increasing concentrations (1, 2, 5, 10, 20, and 50 mM) of acetylcholine (Sigma Chemical Co., Poole, UK), at 15-min intervals between each. Six consecutive breaths of each dilution constituted the challenge dose. Acetylcholine was inhaled using an aerosol delivery control device ( $\mu\text{Med PMS}$ ) driven by compressed air and containing 0.5 ml of the test solution. The mass diameter of the particles averaged between 3 and 4  $\mu\text{m}$ .

Results are expressed as the provocative dose (PD) of acetylcholine, calculated from the dose–response curve, required to increase the  $R_L$  by 50, 100, 200, or 400 cm water/(liter/s) (PD50, PD100, PD200, or PD400), or to decrease the  $C_{\text{dyn}}$  by 25, 50, or 75 cm water/(liter/s) (PD25, PD50, or PD75).

**Bronchoalveolar cell counts and differentiation.** In separate experiments, sensitized guinea pigs challenged either with saline or ovalbumin and treated with the anti-guinea pig MBP Ig or with its preimmune Ig were anesthetized as above, and a BAL was performed (16). Differential cell counts were performed on cytopspin preparations (Hettich Universal, Tuttingen, Germany) by counting 300 cells after staining with Diff-Quik stain (Baxter Dade AG, Duedingen, Switzerland). The results are expressed as the concentration of eosinophils per milliliter of BAL fluid. Aliquots of 1 ml of the remaining lavage fluid were centrifuged at 200 g for 15 min at 4°C, and the supernatant was collected and stored at  $-20^\circ\text{C}$  until the determination of MBP, as described below.

**MBP determination by ELISA.** Guinea pig eosinophil MBP was purified, reduced, alkylated, and dialyzed, according to a modification of the procedure of Gleich et al. (17).

For the determination of MBP levels in the supernatants of BAL fluid, the samples were alkylated and reduced before the assay to avoid polymerization of the MBP molecule, and ELISA was performed using the mouse mAbs 8A12 and 8D12 against guinea pig MBP (kindly provided by Dr. G. Sturton, Bayer plc, Slough, Berkshire, UK), as previously described (15, 18). Briefly, microtiter plates (96-well Immuno Plate MaxiSorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with the mAb 8A12 in coating buffer, and nonspecific binding was blocked by the addition of 1% BSA. Dilutions of standard reduced and alkylated purified guinea pig MBP were then added in concentrations ranging from 1 to 1,000 ng/ml and incubated overnight at 4°C. The biotinylated mAb, 8D12, at 1:400 (18) was added, and plates were incubated at room temperature for 2 h. The avidin–biotin–horseradish peroxidase complex, at 1:100, was then added for 30 min. Finally, 100  $\mu\text{l}$  of freshly prepared substrate solution (TMB microwell peroxidase substrate; KPL Inc., Gaithersburg, MD) was added to each well. After 10 min of incubation, the reaction was stopped by the addition of 2 M HCl. Absorbance was read at 450 nm with an automatic microplate reader (model MR 5000; Dinattech Laboratories, Saint-Cloud, France). The lower detection limit of the assay was  $\sim 10$  ng MBP/ml sample.

The performance of the purified rabbit anti-guinea pig MBP Ig was evaluated using an indirect ELISA. Briefly, dilutions (1:1,000 or 1:2,000) of anti-MBP-purified Ig were incubated for 2 h at room temperature instead of the 8D12 mAb. 100  $\mu\text{l}$  of goat peroxidase-labeled anti-rabbit IgG (Sera-Lab, Crawley Down, UK), at 1:4,000, was then added to each well. After 30 min at room temperature, the enzyme substrate was added as above, and absorbance was measured after 20 min at 450 nm.

Rabbit preimmune Ig (dilutions up to 1:200) were used as a control to check the specificity of the assay.

**Statistical analysis.** Results are expressed as mean  $\pm$  SEM of the indicated number of experiments. One-way ANOVA was used to determine significance among the groups. If a significant variance was found, an unpaired Student's *t* test was used to assess comparability between means. A value of  $P \leq 0.05$  was considered significant.

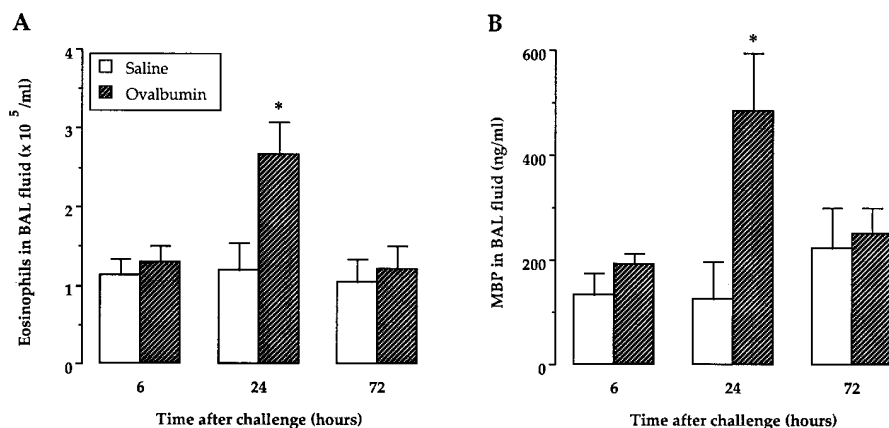
## Results

**Kinetics of eosinophil accumulation and MBP release in the BAL fluid.** Ovalbumin inhalation by sensitized guinea pigs was followed by a significant increase in the number of eosinophils in the BAL fluid at 24 h (Fig. 1). No changes were detected at 6 h (Fig. 1). 72 h after antigen stimulation, eosinophil counts returned to values observed in saline-challenged animals (Fig. 1).

Detectable concentrations of MBP were measured in the supernatant of BAL fluid from sensitized guinea pigs, irrespective of the challenge, i.e., saline or ovalbumin, or of the time point selected, i.e., 6, 24, or 72 h (Fig. 1).

A kinetics study showed a significant elevation in the BAL contents of MBP 24 h after antigen challenge (Fig. 1). Indeed,  $124.6 \pm 73.0$  and  $484.0 \pm 112.0$  ng/ml MBP was found in the supernatant of BAL fluid of saline- and ovalbumin-challenged guinea pigs, respectively ( $P < 0.05$ ,  $n = 6$ ).

**Antigen capture ELISA.** Fig. 2 illustrates a typical standard curve obtained with the double sandwich ELISA for guinea pig MBP using a combination of 8A12 and biotinylated 8D12 mAbs. Results plotted on a logarithmic scale indicate that optimal measurements cover a range of 10–1,000 ng/ml MBP. Using the assay protocol described above and the combinations of 8A12 and purified rabbit anti-guinea pig MBP Ig



**Figure 1.** Kinetics of antigen-induced eosinophil accumulation (A) and MBP release (B) in the BAL fluid from sensitized guinea pigs. Animals were challenged with aerosolized saline or ovalbumin, and BAL was performed 6, 24, or 72 h thereafter. Eosinophil numbers were determined on cytopsin preparations after staining with Diff-Quik stain, and MBP was measured in the cell-free supernatant of BAL fluid by a specific double sandwich ELISA. Results are means  $\pm$  SEM of six experiments. \* $P < 0.05$  compared with saline-challenged guinea pigs.

at 1:1,000 (Fig. 2) or 1:2,000 (data not shown), we observed a similar optimal range of measurements.

**Kinetics of bronchial hyperreactivity.** Ovalbumin inhalation by sensitized guinea pigs was followed by an enhanced response to aerosolized acetylcholine at 72 h, a phenomenon expressed by a significant decrease in the PD200 and PD50 values for  $R_L$  and  $C_{dyn}$ , respectively (Fig. 3). Bronchial reactivity to acetylcholine was not significantly modified 6 and 24 h after antigen challenge (data not shown). Consequently, the time point of 72 h was selected to study the effect of the anti-MBP Ig.

**Effect of anti-MBP Ig on antigen-induced bronchial hyperreactivity and eosinophil accumulation in BAL fluid.** The baseline bronchial resistance to inflation was not significantly different between saline- and ovalbumin-challenged untreated and control or anti-MBP Ig-treated guinea pigs, used 72 h after the challenge. Indeed,  $R_L$  values of  $79.2 \pm 6.8$ ,  $93.6 \pm 13.5$ ,  $82.3 \pm 7.4$ , and  $83.7 \pm 11.3$  cm water/(liter/s) and values of  $C_{dyn}$  of  $1.40 \pm 0.02$ ,  $1.30 \pm 0.10$ ,  $1.40 \pm 0.12$ , and  $1.71 \pm 0.20$  cm water/(liter/s) were found in saline- and ovalbumin-challenged untreated, or control- or anti-MBP Ig-treated guinea pigs, respectively ( $n = 4-6$ , differences not statistically significant).

Antigen challenge by sensitized untreated- or control pre-immune Ig-treated guinea pigs resulted in an augmented bronchoconstrictor response to inhaled acetylcholine at 72 h, as shown by a significant decrease in PD50-PD400 values for  $R_L$  and of PD25-PD75 values for  $C_{dyn}$  compared with saline-challenged animals (Fig. 3). The intranasal administration of anti-MBP Ig 1 h before and 5 h after antigen stimulation suppressed bronchial hyperreactivity to acetylcholine (Fig. 3). Treatment with anti-MBP Ig or with preimmune rabbit Ig did not affect the intensity of the bronchial responses to acetylcho-

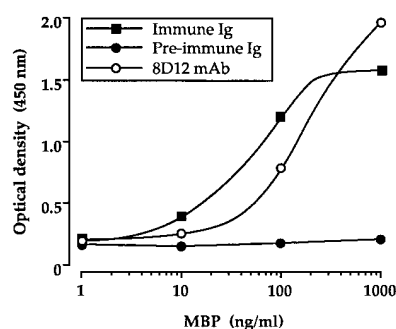
line at 24 h (data not shown). In separate experiments, the intranasal instillation of anti-MBP Ig failed to modify BAL eosinophilia observed 24 h after ovalbumin inhalation. Indeed,  $2.67 \pm 0.41$ ,  $2.72 \pm 0.67$ , and  $2.20 \pm 0.38 \times 10^5$  eosinophils/ml were enumerated in BAL fluid from ovalbumin-challenged untreated, preimmune Ig-, or anti-MBP Ig-treated animals, respectively ( $n = 5-6$ , differences not statistically significant).

## Discussion

The concept that eosinophils participate in the development and perpetuation of bronchial asthma is largely accepted (4, 13). In particular, a role for MBP, the best-characterized eosinophil-derived cationic protein, in the establishment of bronchial hyperreactivity has been suggested (19). In addition, morphologic analysis of BAL fluid from asthmatic patients showed the presence of degranulated eosinophils (1, 3), and immunofluorescence studies have demonstrated MBP deposition in the bronchial tissue of patients who died from status asthmaticus (20). Finally, the levels of MBP in blood and BAL fluid from asthmatics have been correlated with the severity of the symptoms (13, 14).

In the present study, we show that multiple antigen inhalations by aerosol-sensitized guinea pigs result in an accumulation of activated eosinophils in the airways, as demonstrated by the rise in the levels of MBP in the cell-free supernatant of BAL fluid. These phenomena are followed by an increase in the bronchial reactivity to inhaled acetylcholine. At the time of bronchial hyperreactivity, the number of eosinophils and the level of MBP had returned to basal values, indicating a temporal dissociation between changes in bronchopulmonary functions and leukocytic inflammation.

We have already postulated the requirement of local eosinophil activation and subsequent release of cationic proteins for the acquisition of bronchial hyperreactivity in the guinea pig (15). Parallel studies demonstrating the ability of exogenously administered MBP to increase the *in vitro* and *in vivo* contractile response of the bronchial smooth muscle of different species further support this hypothesis (9-12). In addition to MBP, another eosinophil cationic protein, namely eosinophil peroxidase, was shown to elicit bronchial hyperreactivity in nonhuman primates (11), suggesting that both these cationic proteins are capable of altering directly the bronchopulmonary function. However, the formal recognition that *in vivo* inhibition or neutralization of locally released eosinophil-derived



**Figure 2.** Standard curve of guinea pig MBP obtained using the combination of the mouse anti-guinea pig MBP mAb 8A12 and, as a second antibody, either the 8D12 mAb at 1:400 or the purified rabbit anti-guinea pig Ig at 1:1,000, or its control preimmune rabbit Ig at 1:200.

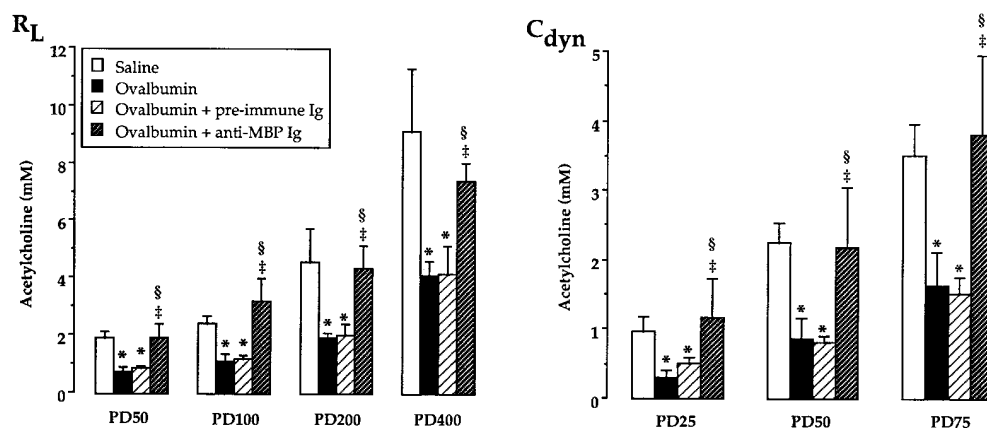


Figure 3. Effect of the anti-MBP Ig of antigen-induced bronchial hyperreactivity to inhaled acetylcholine in sensitized guinea pigs. Sensitized guinea pigs were challenged by aerosolized saline or ovalbumin, and they were anesthetized and ventilated 72 h after the challenge. Acetylcholine (1–50 mM at 15-min intervals) was inhaled, and changes in air-flow and transpulmonary pressure were measured. Animals were either untreated or treated by the intranasal route, with 200  $\mu$ l of purified rabbit anti-guinea pig MBP Ig at 2.5 mg/ml,

or with the same amount of control preimmune Ig, 1 h before and 5 h after antigen challenge. Results are expressed as the provocative dose of acetylcholine, calculated from the dose–response curve, required to increase the  $R_L$  by 50, 100, 200, or 400 cm water/(liter/s) (PD50, PD100, PD200, or PD400) or to decrease the  $C_{dyn}$  by 25, 50, or 75 cm water/(liter/s) (PD25, PD50, or PD75). Results are means  $\pm$  SEM of 6–8 experiments. \* $P$  < 0.05 compared with saline-challenged guinea pigs; † $P$  < 0.05 compared with ovalbumin-challenged untreated guinea pigs. § $P$  < 0.05 compared with ovalbumin-challenged preimmune Ig-treated guinea pigs.

cationic proteins, particularly MBP, would prevent allergic bronchopulmonary hyperreactivity was still missing. Circumstantially, heparin, which binds to cationic proteins (21), has been shown to inhibit experimental bronchial hyperreactivity (22). Here, we bring evidence that the intranasal treatment of sensitized guinea pigs with purified rabbit Igs raised against guinea pig MBP, given 1 h before and 5 h after antigen challenge, suppresses the increased airway response to inhaled acetylcholine that develops at 72 h. This suggests that secretion of MBP by activated eosinophils is a key event for the subsequent acquisition of bronchial hyperreactivity in this model.

In the present study, the ability of the rabbit Igs to bind guinea pig MBP has been documented using an indirect ELISA and the specificity has been demonstrated using the preimmune rabbit Igs.

Damage of the respiratory epithelium is believed to be the main mechanism by which eosinophil-derived cationic proteins, and particularly MBP, are responsible for the increase in nonspecific bronchial hyperreactivity frequently observed in asthmatic patients (6–8, 19). However, despite an intense eosinophil infiltration and an increase in the levels of MBP 24 h after antigen exposure, we could not demonstrate histologically any epithelial damage or exfoliation in the bronchial mucosa of ovalbumin- as opposed to saline-challenged animals (Lapa e Silva, personal communication). These results are reminiscent of those of Uchida et al. (12), who demonstrated that the intratracheal instillation of high doses of MBP to rats was followed by bronchial hyperreactivity to inhaled methacholine without histological modifications of the epithelium. Together, these findings suggest that exogenously administered MBP or MBP secreted in the airways upon antigen challenge may alter bronchopulmonary function by mechanisms independent of epithelial damage. These mechanisms include alterations in epithelial function, such as the induction of the release of bronchoconstrictor mediators and/or a decreased availability of epithelium-derived relaxing factor (23). In addition, MBP may activate sensory C fibers, as shown in rats where the administration of two synthetic cationic proteins, poly-L-lysine or poly-L-arginine, elicited a tachykinin-dependent bronchial hyperreactivity to methacholine (24). Coyle et al. (25) recently

demonstrated that in vivo MBP-induced bronchial hyperreactivity in rats paralleled a rise in the levels of immunoreactive kinin and kallikrein-like activities in the BAL fluid. Bronchial hyperreactivity was prevented by the pretreatment of the animals with a selective bradykinin type 2 receptor antagonist, suggesting that at least some of the effects of MBP may be mediated by the generation of immunoreactive kinins.

Interestingly enough, Fryer and Wills-Karp (26) have demonstrated that the impairment of the inhibitory  $M_2$  muscarinic autoreceptor function on parasympathetic nerves of lung of antigen-challenged guinea pigs results in in vivo airway hyperresponsiveness to electrical stimulation of the vagus. This phenomenon was reversed by heparin and poly-L-glutamate (27), which bind to and neutralize MBP in vitro (21), suggesting that endogenously released cationic proteins, particularly MBP, may increase bronchial reactivity by antagonizing the  $M_2$  muscarinic receptor.

Cationic proteins also promote airway vascular leakage (24, 28), which, in turn, may alter epithelial permeability and facilitate the access of inhaled agonist to the smooth muscle, leading to an amplification of the bronchial responses. However, the inhibition of bronchial hyperreactivity by rabbit anti-guinea pig MBP Igs we reported here is unlikely to result from a blockade of vascular leakage, since changes in acetylcholine-induced bronchoconstriction were observed at a late time point (72 h) after antigen challenge, when increase in vascular permeability has already resolved.

Incubation of mast cells or basophils with MBP results in a noncytotoxic histamine release (29). More recently, some T cell-derived eosinophilotactic cytokines such as IL-3, IL-5, and GM-CSF have been shown to enhance MBP-induced mediator release from human basophils (30). Together, these findings suggest that neutralization of MBP secreted by activated eosinophils may also attenuate the release of bronchoconstrictor and inflammatory mediators by other cell types present in their environment.

In conclusion, our findings demonstrate that, as in human asthma, antigen challenge in aerosol-sensitized guinea pigs elicits eosinophil activation in the airways and bronchial hyperreactivity to inhaled acetylcholine. Bronchial hyperreactivity

ity is suppressed by treating the animals with purified rabbit IgG directed against guinea pig MBP, suggesting that therapeutic interventions designed specifically to neutralize MBP secreted in the airways during allergic reactions may inhibit alterations in the bronchopulmonary function.

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