



Structural and functional localization of airway effects from episodic exposure of infant monkeys to allergen and/or ozone

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Abstract

Both allergen and ozone exposure increase asthma symptoms and airway responsiveness in children. Little is known about how these inhalants may differentially modify airway responsiveness in large proximal as compared to small distal airways. We evaluated whether bronchi and respiratory bronchioles from infant monkeys exposed episodically to allergen and/or ozone differentially develop intrinsic hyperresponsiveness to methacholine and whether eosinophils and/or pulmonary neuroendocrine cells play a role. Infant monkeys were exposed episodically for 5 months to: (1) filtered air, (2) aerosolized house dust mite allergen, (3) ozone 0.5 ppm, or (4) house dust mite allergen + ozone. Studying the function/structure relationship of the same lung slices, we evaluated methacholine airway responsiveness and histology of bronchi and respiratory bronchioles. In bronchi, intrinsic responsiveness was increased by allergen exposure, an effect reduced by bombesin antagonist. In respiratory bronchioles, intrinsic airway responsiveness was increased by allergen + ozone exposure. Eosinophils were increased by allergen and allergen + ozone exposure in bronchi and by allergen exposure in respiratory bronchioles. In both airways, exposure to allergen + ozone resulted in fewer tissue eosinophils than did allergen exposure alone. In bronchi, but not in respiratory bronchioles, the number of eosinophils and neuroendocrine cells correlated with airway responsiveness. We conclude that episodically exposing infant monkeys to house dust mite allergen with or without ozone increased intrinsic airway responsiveness to methacholine in bronchi differently than in respiratory bronchioles. In bronchi, eosinophils and neuroendocrine cells may play a role in the development of airway hyperresponsiveness.

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Introduction

Asthma in young children has been increasing over the last four decades (Anderson, 2005). It is a complex phenotype involving an interaction of genetic and environmental factors (Taussig et al., 2003). Recently, attention has been directed at what may contribute to the development of asthma in very early life with the hope that preventive interventions can change the course of the disease (Taussig et al., 2003; Brussee et al., 2005; Guilbert et al., 2004).

When adult asthmatics are repeatedly exposed to allergen (Arshad et al., 1998) or ozone (Gong and Linn, 1997), airway responsiveness to methacholine is increased. Similar experimental studies have not been performed in children, but epidemiologic studies of children show that ambient ozone exposure is associated with the development of asthma (McConnell et al., 2002) and ozone plus allergen exposure results in more asthma symptoms and inhaler use than ozone exposure alone (Delfino et al., 1996).

The mechanisms by which repeated exposure to ozone and allergen increase airway hyperresponsiveness are not completely understood. Some of the mechanisms probably include systemic effects such as nerve activation (Joad et al., 1998; Chen et al., 2003) or release of mediators from cells in the

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airway lumen or circulation. However, some of the mechanisms may be intrinsic to the airways. Two resident cell types, eosinophils and pulmonary neuroendocrine cells (PNECs) may be involved in these local effects. The number of eosinophils in the airways has been shown to be increased in allergic asthma in children (Lemanske, 2002). Eosinophils are increased by ozone exposure in guinea pigs and appear to mediate methacholine hyperresponsiveness 3 days later (Yost et al., 2005). Less studied are PNECs, cells that are increased in number in asthma (Aguayo, 1993) and contain bronchoconstrictors including bombesin-like or gastrin-related peptide (BLP/GRP) (Lach et al., 1993).

How allergen or ozone affects the physiology of airways of different airway generations is not understood. Specifically, we have only recently appreciated the importance of understanding the structure and function of the smallest airways in asthma. Postmortem examination shows extensive disease in small airways in asthma; however, these airways have been poorly evaluated due to difficulty of in vivo sampling and lack of specificity of physiologic measurements (Howarth, 1998).

Our group has been collaboratively addressing the role of allergen and/or ozone on the development of asthma in infant rhesus monkeys. Among other findings, we have shown that infant monkeys exposed episodically to allergen and ozone develop an increase in airway resistance and airway responsiveness to histamine in vivo, as well as an increase in eosinophils in bronchoalveolar lavage fluid at 6 months of age (Schelegle et al., 2003). In this report, we used videomicroscopy of airway slices in conjunction with morphometry of the same slices to test the hypothesis that episodic allergen and/or ozone exposure in vivo would affect intrinsic airway responsiveness in vitro differently in the bronchi than in the respiratory bronchioles and that eosinophils and/or PNECs would play a role.

Methods

All protocols were approved by the Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act and Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Exposures. The exposures were designed to mimic the episodic exposures experienced by human infants and have been previously described (Schelegle et al., 2003). Briefly, 23 infant rhesus macaques (*Macaca mulatta*) monkeys born over two seasons at the California National Primate Research Center were studied. Care and housing of animals complied with the provisions of the Institute of Laboratory Animal Resources and conformed to practices established by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Infant monkeys to be exposed to aerosolized house dust mite allergen (HDMA, $435 \pm 96 \mu\text{g}/\text{m}^3$ protein (mean $\pm$ SD), *Dermatophyoides farinae*, Greer Laboratories, Inc. Lenoir, NC, $n = 12$) were sensitized by subcutaneous injection of HDMA in alum and intramuscular injection of heat-killed *Bordetella pertussis* cells at 14 and 28 days of age. Sensitization was confirmed by a positive intradermal skin test to HDMA on day 38 of the exposure protocol in 11 of 12 sensitized infants (Schelegle et al., 2003). At 1 month of age, the monkeys were randomly assigned to receive 11 cycles of exposure to either (1) filtered air (FA), (2) allergen, 2 h/day, 3 days on and 11 days off, (3) ozone (O_3) 0.5 ppm for 6 h/day, 5 days on and 9 days off, or (4) allergen + O_3 (allergen exposure on last 3 days of O_3 exposure but not at the same time of day). The studies were performed 3–5 days after the last exposure.

Ozone was generated as previously described (Schelegle et al., 2003) and monitored using a Dasibi 1003-AH ozone analyzer (Dasibi Environmental

Corporation, Glendale, CA). *Dermatophyoides farinae* was diluted in phosphate-buffered saline and aerosolized with a nebulizer (Miniheart, Westmed, Inc., Tucson, AZ) as previously described (Schelegle et al., 2003).

Airway responsiveness using videomicroscopy. Airway slices for videomicroscopy were prepared for all monkeys from both years of the study (5–6 animals/group). As we have done previously (Kott et al., 2002), each monkey was initially anesthetized with ketamine (10 mg/kg i.m.), deeply anesthetized with pentobarbital ($>22 \text{ mg}/\text{kg}$), ventilated, then euthanized with pentobarbital (44 mg/kg) and exsanguinated. The heart–lung–tracheal unit was removed and the right accessory lobe cannulated and warm (37 °C) SeaPlaque agarose (1.25%, FMC Bioproducts Rockland, ME) in Waymouth's medium (Gibco BRL, Life Sciences, Grand Island, NY, pH 7.40) slowly instilled. After 30 min in ice-cold (4 °C) Waymouth's medium, the lobe was cut into 10 mm blocks that were then cut with a vibratome perpendicular to the main central airway of the lobe into slices that were 650- μm thick for proximal airways and 600- μm thick for distal airways. Each slice was tacked to a cover-slip with small, discrete spots of nexaband glue (Burns Veterinary Supply, Reno, NV) near the pleural edges and placed in 35-mm petri dishes (Corning, NY) in 3 ml ice-cold Waymouth's. Only those airways with edges completely in focus, with no airway branching, and in a perpendicular plane (Kott et al., 2002) were chosen for methacholine challenge. The agarose plug from each airway was removed prior to study by gently probing it using a 32-gauge needle. Images of the cross-sectional area of the main axial airway path were captured using an Olympus BH2 light microscope with water-immersion lenses connected to a Apple Macintosh computer running NIH Image (NIH, Bethesda, MD) software via a DAGE MTI video camera.

The slices were stabilized for a minimum of 30 min with Kreb's Henseleit buffer (Sigma Chemicals, St. Louis, MO) followed by Kreb's Henseleit buffer changes every 5 min until the airway lumen was changing less than 10% between washes (minimum of 8 changes). All solutions were kept at 37 °C and aerated with 95% O_2 and 5% CO_2 at pH 7.35 ± 0.03 . Increasing concentrations of methacholine (Sigma Chemicals, St. Louis, MO) in Kreb's Henseleit buffer (10^{-9} M to 10^{-4} M) were then added for 5 min each and an image was then captured. Because bombesin is a PNEC mediator and can cause bronchoconstriction, the bombesin antagonist ([Leu¹³-(ψ -CH₂NH)-Leu¹⁴]-Bombesin, 100 nM, Research Biochemicals International, Natick, MA) was included in the incubation buffer. Control slices received the equivalent number of changes with Kreb's Henseleit buffer only.

Histologic and morphometric evaluation of the airways. Following measurement of airway response, lung slices were fixed in 1% paraformaldehyde and embedded in paraffin (Paraplast-20, Oxford Labware, St. Louis, MO). Due to technical problems with the fixative, the slices from the FA and HDMA groups from year one could not be studied. Serial tissue sections 5- μm thick were prepared from each lung slice. A bronchus was identified by the presence of cartilage in the wall, a bronchiole by the lack of cartilage or alveolar outpocketings within the wall, and a respiratory bronchiole by alveolar outpocketings along the wall.

Eosinophils in both bronchi and respiratory bronchioles were counted using paraffin sections stained with Combined Eosinophil/Mast Cell Stain (American Master Tech Scientific, Inc., Lodi, CA). The number of eosinophil profiles in the airway wall per basal lamina length of the airway mucosa was counted. In the respiratory bronchioles, the alveolar outpocketings were not measured.

PNECs in both bronchi and respiratory bronchioles were stained with anti-serotonin (5-hydroxytryptamine, 5-HT, Sigma, St Louis, MO) made in rabbit using the following immunohistochemical procedure. Paraffin sections were taken through xylene and ethanol to remove the paraffin and treated with AR-10 antigen retrieval solution (BioGenex, San Ramon, CA). Endogenous peroxidase was blocked with 3% H_2O_2 , slides washed in phosphate-buffered saline, and 15% normal goat serum applied as a nonspecific blocking step. Sections were incubated in anti-5-HT antibody for 1 h at room temperature, then washed in phosphate-buffered saline. Sections were subsequently incubated in biotinylated secondary antibody, Vectastain Elite ABC reagent and DAB substrate kit (Vector Laboratories, Inc., Burlingame, CA) for peroxidase according to the manufacturers directions. Sections were counterstained with Hematoxylin (Vector Laboratories, Inc., Burlingame, CA). PNECs including those occurring in

clusters as pulmonary neuroepithelial bodies were imaged and the area was measured using NIH image software. The basal lamina length for each airway was also determined using NIH image software. From these measurements and the known thickness of each section, the volume of PNECs per basal lamina surface area was calculated for each airway.

Data analysis and statistical evaluation. For videomicrometry, the epithelial luminal border of the airway was traced, and the luminal area was calculated using NIH Image software. The data were expressed as a percent of the lumen measured in the last buffer wash. Concentration response curves were compared using a repeated measures analysis of variance. Each slice was considered a separate data point. One to 3 slices from proximal and distal airways were included from each animal. When appropriate, post hoc analyses consisted of a series of Scheffe contrast tests among the treatment groups (SAS/Stat, SAS Institute, Cary, NC). Data were log transformed if variances differed by more than 3-fold. Differences in eosinophils and PNECs between exposure groups were determined by a one-way ANOVA. A linear regression (Excel, Microsoft Corporation, Redmond WA) was used to determine whether there was a linear relationship between eosinophils or PNECs and airway responsiveness and between eosinophils and PNECs. Airway responsiveness was defined as the PC_{50} , which was the concentration of methacholine that decreased the luminal area to 50% of its area at the highest concentration of methacholine (-4.0 log M). Significance was defined as $P < 0.05$.

Results

Morphology of airways

All airways could be divided into bronchi (with cartilage present) and respiratory bronchioles (alveolar outpocketings present). There was no difference in baseline luminal area between exposure groups in bronchi (182 ± 21 FA, 205 ± 16 HDMA, 246 ± 27 O_3 , 211 ± 20 HDMA + $O_3 \times 10^3 \mu m^2$) or in respiratory bronchioles (82.6 ± 12.9 FA, 85.6 ± 7.8 HDMA, 78.6 ± 5.6 O_3 , 68.9 ± 7.6 HDMA + $O_3 \times 10^3 \mu m^2$, mean $\pm$ SEM).

Effect of exposures on airway responsiveness to methacholine

Airway responsiveness to methacholine was increased by HDMA in bronchi and by HDMA + O_3 in respiratory bronchioles (Fig. 1). In bronchi from one allergen-exposed infant, [Leu¹³-(Ψ -CH₂NH)-Leu¹⁴]-bombesin decreased the hyperresponsiveness to methacholine (Fig. 2). This bombesin

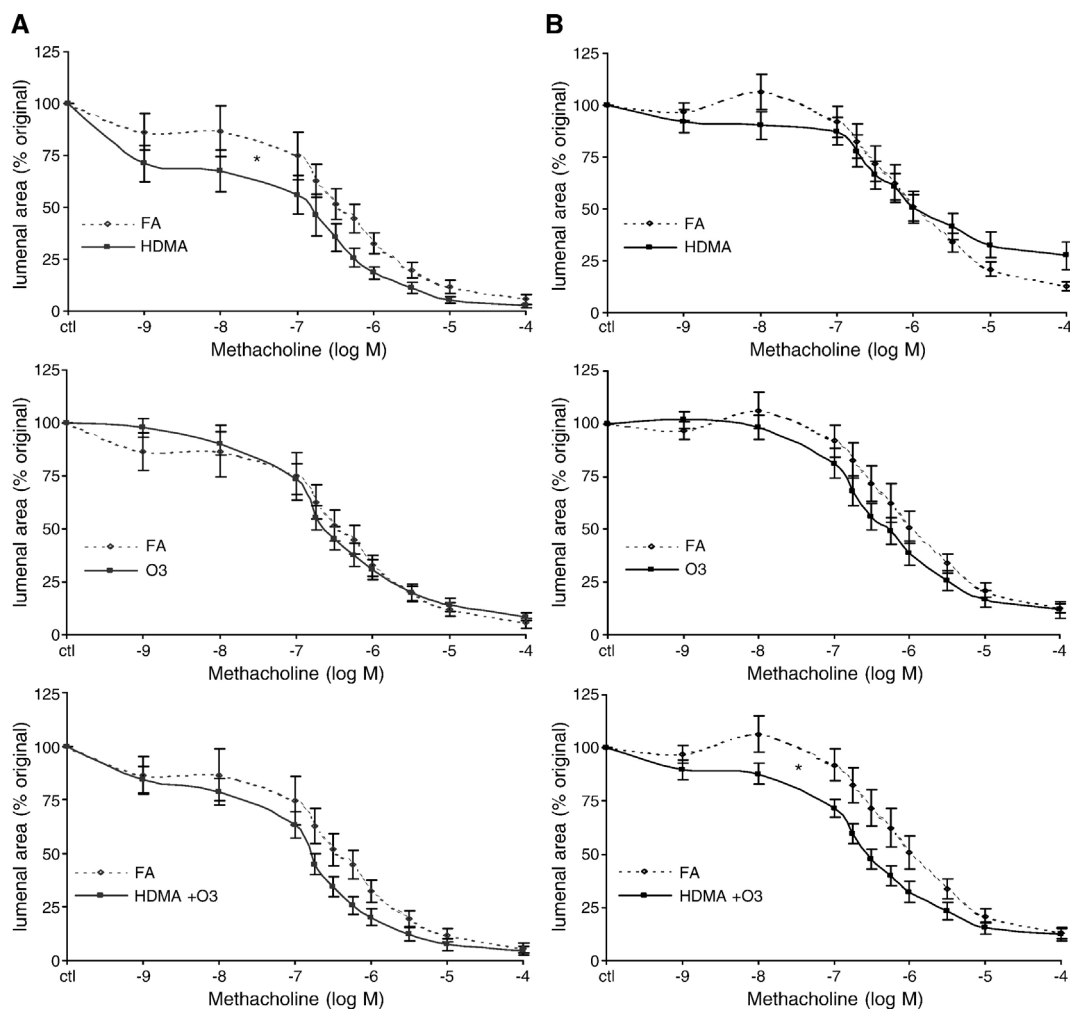


Fig. 1. Airway responsiveness of (A) bronchi and (B) respiratory bronchioles from infant monkeys exposed episodically to filtered air (FA) compared with house dust mite allergen (HDMA, top panel, 13 slices, 5 animals for bronchi and bronchioles), ozone (O_3 , middle panel, 13 slices, 6 animals for bronchi; 14 slices, 6 animals for bronchioles), or HDMA + O_3 (bottom panel, 14 slices, 6 animals for bronchi; 12 slices, 5 animals for bronchioles). FA controls had 12 slices, 6 animals in bronchi; 9 slices, 5 animals for bronchioles. Airway responsiveness was increased by episodic exposure to HDMA in bronchi ($P = 0.037$) and by episodic exposure to HDMA + O_3 in respiratory bronchioles ($P = 0.049$). Values are means $\pm$ SEM.

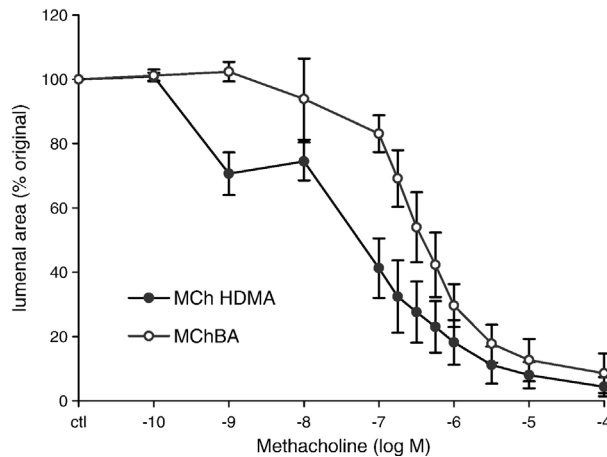


Fig. 2. Effect of bombesin antagonist on responsiveness of bronchi from one infant monkey episodically exposed to house dust mite allergen (HDMA). The bombesin antagonist ([Leu¹³-(ψ-CH₂NH)-Leu¹⁴]-bombesin, BA) reversed the hyperresponsiveness due to exposure to HDMA (2 slices each, $P = 0.001$). Values are means $\pm$ SEM.

receptor antagonist had no effect on baseline airway caliber, on respiratory bronchiolar responsiveness, or on bronchial responsiveness in any of the other exposure groups. In control slices, repeated administration of Kreb's Henseleit buffer alone did not change airway luminal area.

Evaluation of eosinophils

The number of eosinophils in bronchi was increased by HDMA and the HDMA + O₃ exposure (Fig. 3A). In respiratory bronchioles, the number of eosinophils was increased by HDMA exposure, but not by HDMA + O₃ exposure (Fig. 3B). In both bronchi and respiratory bronchioles, HDMA + O₃ exposure resulted in fewer eosinophils than did HDMA exposure alone. In bronchi, across all exposures, there was a correlation between airway responsiveness and number of eosinophils such that the lower the PC₅₀ (the greater the airway responsiveness), the greater the number of eosinophils ($r^2 = 0.26$, $P = 0.005$). In respiratory bronchioles, there was no correlation.

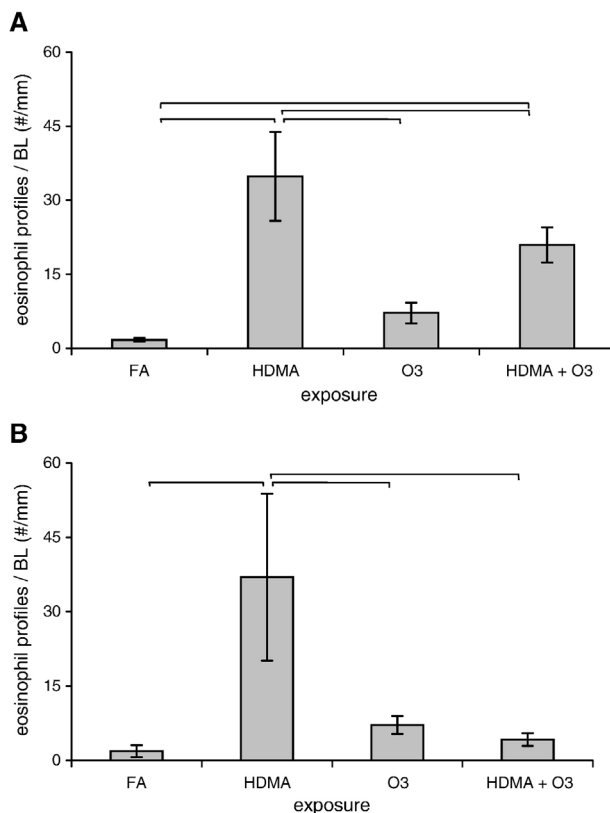


Fig. 3. (A) The number of epithelial eosinophils per basal lamina of bronchi from infant monkeys exposed episodically to (1) filtered air (FA, 4 slices, 3 animals), (2) house dust mite allergen (HDMA, 3 slices, 2 animals), (3) ozone (O₃, 10 slices, 5 animals), or (4) HDMA + O₃ (12 slices, 6 animals). (B) The number of epithelial eosinophils per basal lamina of respiratory bronchioles from infant monkeys exposed episodically to (1) FA (4 slices, 3 animals), (2) HDMA (3 slices, 2 animals), (3) O₃ (11 slices, 6 animals), or (4) HDMA + O₃ (11 slices, 6 animals). The number of epithelial eosinophils was increased by HDMA and by HDMA + O₃ in the bronchi ($P = 0.001$) and by HDMA in the respiratory bronchioles ($P < 0.007$). In both airways, HDMA + O₃ decreased the number of epithelial eosinophils found with HDMA alone. Brackets indicate $P < 0.05$ Fisher LSD. Values are means $\pm$ SEM.

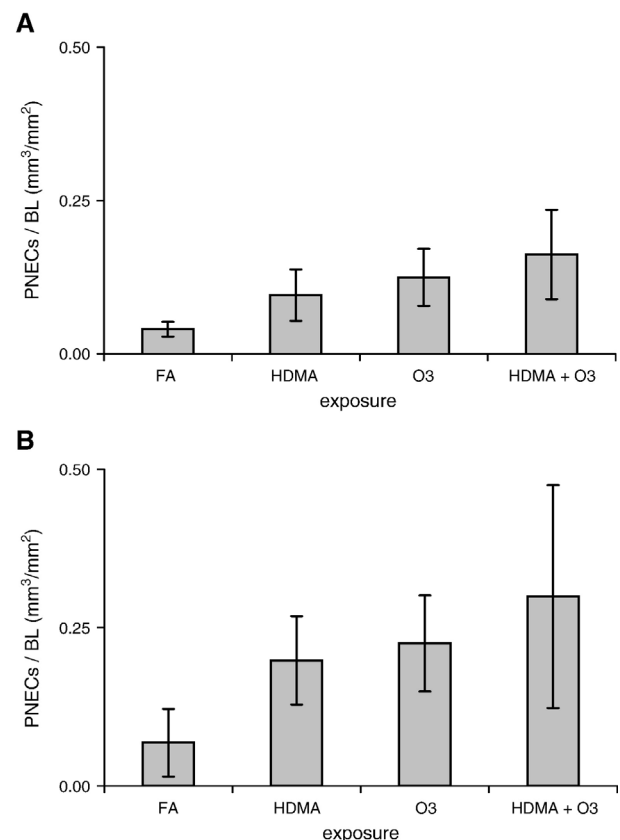


Fig. 4. (A) The pulmonary neuroendocrine cell (PNEC) volume per surface area basal lamina of bronchi from infant monkeys exposed episodically to (1) filtered air (FA, 4 slices, 3 animals), (2) house dust mite allergen (HDMA, 4 slices, 2 animals), (3) ozone (O₃, 13 slices, 6 animals), or (4) HDMA + O₃ (13 slices, 6 animals). (B) The PNEC volume per surface area basal lamina of respiratory bronchioles from infant monkeys exposed episodically to (1) FA (4 slices, 3 animals), (2) HDMA (4 slices, 2 animals), (3) O₃ (12 slices, 6 animals), or (4) HDMA + O₃ (12 slices, 6 animals). Although there was a trend for the PNEC area to increase with the exposures, the changes were not statistically significant. Values are means $\pm$ SEM.

Evaluation of PNECs

The pulmonary neuroendocrine cell area tended to increase with the exposures but the effect was not statistically significant (Fig. 4). In bronchi, across all exposures, there was a correlation between the airway responsiveness and PNEC area such that the lower the PC₅₀ (the greater the airway responsiveness), the greater the area of PNECs ($r^2 = 0.33$, $P = 0.0004$). In the respiratory bronchioles, there was no correlation.

Relationship between eosinophils and PNECs

There was no correlation between the number of eosinophils and PNEC area in the bronchi.

Discussion

This study showed that exposing infant monkeys episodically to allergen or allergen plus ozone increased intrinsic airway responsiveness to methacholine differently in bronchi than in respiratory bronchioles. In bronchi, allergen alone made the airways hyperresponsive but in respiratory bronchioles, both allergen and ozone together were required to produce the effect. In bronchi, both PNECs and eosinophils appeared to play a role in these effects. Both PNEC area and eosinophil number correlated with airway reactivity. In addition, for PNECs, the bombesin receptor antagonist blocked the increase in airway responsiveness due to allergen exposure.

In this model, there was a trend for allergen exposure to affect the large airways (Fig. 1A, top panel, $P = 0.036$, and bottom panel, $P = 0.12$) whereas ozone exposure affected the respiratory bronchioles (Fig. 1B, middle panel, $P = 0.26$, and bottom panel, $P = 0.049$). Conversely, ozone alone ($P = 0.96$) did not affect the large airways and allergen alone ($P = 0.96$) did not affect the respiratory bronchioles. In the same animals in vivo, only allergen plus ozone exposure increased airway responsiveness (Schelegle et al., 2003). The trend seen in the large airways (Fig. 1A, bottom panel) and the statistically significant effect in the respiratory bronchioles (Fig. 1B, bottom panel) seen with allergen plus ozone exposure support the hypothesis that intrinsic airway responsiveness explains some of the in vivo findings. However, the allergen effect in bronchi was not demonstrable with our in vivo studies. It may be that in addition to the local effects on intrinsic airway reactivity, ozone exposure caused neural effects not seen with allergen exposure. We have shown that extended exposure to ozone increases responsiveness of airway nerves (Joad et al., 1998) and neurons in the respiratory region of the brainstem (Chen et al., 2003).

Since airway resistance increases by the inverse of the radius to the fourth power, our changes in responsiveness are physiologically important. For instance, in the FA-exposed animals, the bronchial resistance would have increased by 9.4-fold at a methacholine concentration of 10^{-6} M, while in the HDMA-exposed animals, it would have increased by 29-fold at the same concentration of methacholine. Similarly, in the FA-exposed animals, the respiratory bronchiolar resistance would have increased by 3.9-fold at a methacholine concentration of 10^{-6}

M, while that in the HDMA + O₃-exposed animals would have increased 9.5-fold (these calculations assume a circular airway). Thus, the exposure to HDMA or HDMA + O₃ would have resulted in a 2- to 3-fold increase in airway resistance.

PNECs are primarily thought of as chemoreceptors (Youngson et al., 1993) that release vasoactive substances important in ventilation–perfusion matching. However, many of the vasoactive substances also control airway function. In this study, we showed a trend toward increased PNECs with allergen and/or ozone exposure. This agrees with other studies that have shown that oxidant (Nylen and Becker, 1993; Shenberger et al., 1997) and allergen (Bousbaa et al., 1994) exposure increases PNECs or their granules and with our own study showing an association between increased PNECs and airway hyperresponsiveness due to sidestream smoke exposure in young rats (Joad et al., 1995). More importantly, we were able to show a significant correlation between the PNECs area and airway responsiveness in bronchi and that airway hyperresponsiveness induced by allergen exposure was blocked by a bombesin receptor antagonist in the one animal tested. Together, these data suggest that, in bronchi but not respiratory bronchioles, PNECs and bombesin within them may play a role in airway responsiveness due to allergen exposure.

Because airway responsiveness correlated with eosinophil number, this study adds data supporting a controversial role for the eosinophil in the pathogenesis of asthma. Evidence in support of a role for eosinophils in asthma includes that eosinophilia is found in humans and animal models of asthma and major basic protein released by eosinophils causes airway hyperresponsiveness (Bochner, 2004). Evidence against a role of eosinophils in asthma includes the finding that, in human asthmatics, a monoclonal antibody to IL-5, the major interleukin responsible for terminal differentiation of eosinophils, markedly decreases blood and sputum eosinophils (although less markedly tissue eosinophils) but not the late asthmatic hyperreactivity to allergen (Leckie et al., 2000; Bochner, 2004) and that, in mice, IL-13 antibody can prevent allergen-induced airway hyperresponsiveness without effecting eosinophilia (Wills-Karp et al., 1998). Our study supports a minor role for eosinophils in the proximal airways only.

Exposing infant primates to allergen increased airway wall eosinophils, an effect that was still present but reduced when ozone with allergen was given together. Since the combined exposure group had more eosinophils in the bronchoalveolar lavage fluid (Schelegle et al., 2003), this may represent transmigration of eosinophils from the airway walls to the lumen in the combined exposure group. Our airway slices were washed extensively before study and it is unlikely that eosinophils were in the lumen. Thus, any effect seen was due to the tissue eosinophils. In bronchi, the largest effect (allergen alone) was associated with the highest number of eosinophils.

Because both PNECs and eosinophils were each related to airway hyperresponsiveness in bronchi, we tested whether they were related to each other. Tsukiji (Tsukiji et al., 2004) showed that allergic inflammation in Brown Norway rats increased mRNA beta-CGRP in neuroepithelial bodies. Although we were unable to demonstrate a correlation between the number of

eosinophils and PNEC area, there may have been an association between increases in their mediator production.

The major advantages of this study are the young age of the primates, the study of respiratory bronchioles, the comparison of the physiology of airway generations, the structure/function analysis of each airway, and the collaboration with other investigators studying the same infants. Infants and young animals are only rarely studied, yet it is likely that for many persons, asthma is established at this stage of development (Guilbert et al., 2004). Primates offer a unique advantage over rodents because as in humans, they have one to three generations of respiratory bronchioles rather than a single terminal bronchiole. We have shown that airway responsiveness of the rat terminal bronchioles and the primate respiratory bronchiole are very different (Kott et al., 2002). Our methodology allowed us to compare histologically verified bronchi with respiratory bronchioles, a comparison that cannot be done with airway strips. Finally, we were able to correlate airway responsiveness with histological characteristics of the same slice, allowing for a powerful association of structure and function. Our collaborative approach to these primates will further allow us to consider other possible mechanisms not studied by us a priori. For instance, using micro-dissection and whole mount techniques, Tran et al. (2004) showed an increase in total smooth muscle mass and bundle size but not bundle abundance in the bronchioles from our allergen-exposed infants.

The major disadvantage of our study was the small number of animals studied and the small number of slices available for study. This was necessary due to the expense and the ethics associated with studying primates. Thus, the study was not powered to avoid Type II errors or to study a number of possible mechanisms using receptor antagonists.

In conclusion, episodically exposing infant monkeys to allergen and/or ozone affected airway generations differently. In bronchi, allergen exposure leads to increased airway responsiveness whereas in respiratory bronchioles, the combination of ozone and allergen was required to produce the effect. In bronchi but not in respiratory bronchioles, eosinophils and PNECs correlated with airway responsiveness and may have played a role in their responsiveness.

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