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OZONE INCREASES AIRWAY HYPERREACTIVITY AND MUCUS HYPERPRODUCTION IN MICE PREVIOUSLY EXPOSED TO ALLERGEN

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Acute exacerbations of asthma represent a common clinical problem with major economic impact. Air pollutants including ozone have been shown to contribute to asthma exacerbation, but the mechanisms underlying ozone-induced asthma exacerbation are only partially understood. The present study aimed to develop a mouse model to gain insight into the development of airway hyperresponsiveness (AHR) to methacholine (MCh) in mice after exposure to both allergen and ozone. Mice were exposed for 20 min per day for 10 consecutive days to an aerosol of 1% ovalbumin (OVA) or saline followed by a single 3-h exposure to clean air or 100, 250, or 500 ppb ozone. Ozone induced AHR in mice previously exposed to OVA when compared to non-exposed (saline) control mice. After a 10-d exposure to OVA, a single exposure to a low (100 ppb) ozone concentration was sufficient to induce AHR. The AHR response was associated with goblet-cell metaplasia. Even the lowest concentration of ozone tested, 100 ppb, which may be exceeded in urban environments and in the workplace, resulted in a significant increase in AHR, most prominent 24 h after exposure in the OVA-exposed mice.

Acute exacerbations of asthma are a common clinical problem with major economic impact (Holgate, 2005). Exacerbations are most commonly due to viral infections (Murray et al., 2004), allergen exposure (Murray et al., 2004), and air pollutants (Etzell, 2003). Both epidemiological evidence and experimental evidence suggest that exacerbations of asthma may be induced by ozone, a ubiquitous oxidizing and highly toxic air pollutant generated photochemically from nitric oxides and hydrocarbons (Krewski & Rainham, 2007). Romieu et al. (1995) reported that the number of emergency visits for

asthma were linked to the concentrations of ozone and sulfur dioxide exposures (Bedeschi et al., 2007). Interestingly, the strongest association was seen between 1-h maximum ozone concentration and a 1-d lag in emergency room visits.

Ozone exposure induces oxidant stress in the lung, which may directly or indirectly contribute to the release of mediators and cytokines in the conducting airways (Bhalla, 1999). In addition, ozone exposure likely leads to opening of the normally tight junctions of the airway epithelium, resulting in edema and transport of proteins across the epithelium (Guth et al.,

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1986; Hu et al., 1982). However, the underlying mechanisms for the increased sensitivity to ozone exposure in asthmatics over that observed in nonasthmatic subjects are only partially understood. This knowledge is critical to the pharmacological treatment or prevention of ozone-induced asthma exacerbations. The aim of the present study was to develop an animal model that allows investigation of the effects of ozone on the respiratory tract of allergen-exposed mice in order to reveal potential mechanisms. The goals of this study were to develop an approach where neither allergen alone nor ozone alone resulted in airway hyperresponsiveness (AHR). In contrast to most other published studies, mice in the present study were only exposed to ozone on a single occasion. The lowest concentration included in the present study (100 ppb) corresponds to the 8-h occupational threshold limit value (TLV), which varies from 50 ppb (0.1 mg/m³) to 100 ppb (0.2 mg/m³), depending on the workload (ACGIH, 2007).

METHODS

Animals

Female BALB/c mice, 8–12 wk old, weighing 20–22 g, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed under specific-pathogen-free conditions. The animals were kept on an ovalbumin (OVA)-free diet. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health (Denver, CO).

Protocol for OVA Exposure

Mice were exposed for 20 min per day on 10 consecutive days to a 1% w/v OVA (grade V, Sigma Chemical Co., St. Louis, MO) in saline aerosol. Aerosols were generated from an ultrasonic nebulizer (Omron, Kyoto, Japan). Control mice were exposed to isotonic saline aerosol.

GENERATION OF OZONE AND OZONE EXPOSURE

Mice were exposed to ozone at 100, 250, or 500 ppb or air for 3 h in stainless-steel wire

cages. The actual ozone concentrations deviated less than 10% from the target concentrations, except during the first 5 min of the exposure period, where the ozone concentration briefly exceeded the target concentration by up to 20%. Cages were set inside 240-L laminar flow inhalation chambers. HEPA-filtered room air was passed through these chambers at 25 changes/h. Room temperature was maintained at 20–25°C. Ozone was generated by directing compressed medical-grade oxygen through an electrical discharge ozone generator (Sander Ozonizer, model 25, Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) located upstream of the exposure chamber. The ozone–air mixture was metered into the inlet air stream with mass flow controllers (MKS Instruments, Inc., model 1359C, Andover, MA). Exposure to HEPA-filtered air was conducted in a separate chamber with age- and treatment-matched control animals. Ozone concentrations were continuously monitored at mouse nose levels within the chamber with a photometric ozone analyzer (Advanced Pollution Instrumentation, Inc., model 400A, San Diego, CA) and recorded on a strip-chart recorder. Calibration of the ozone analyzer was performed by the Colorado Department of Public Health and Environment (Denver, CO).

Airway Reactivity

Airway reactivity to inhaled methacholine (MCh, Sigma) was monitored 6 and 24 h after completion of the 3-h ozone exposure period. Pulmonary resistance was assessed as described (Takeda et al., 1997). Anesthetized and intubated mice were initially challenged with saline (baseline), followed by challenge to increasing concentrations of MCh (1.56, 3.125, 6.25, and 12.5 mg/ml). Aerosols of MCh were administered for 8 breaths at a rate of 60 breaths/min, tidal volume of 500 μ l by a second ventilator (model 683; Harvard Apparatus, South Natick, MA). From 1 to 5 min after each aerosol challenge, data of lung resistance and dynamic compliance were continuously collected. The maximum lung response to the concentration of MCh being tested was used, and was expressed as percent change over

saline control. To avoid bias of data, a block design was employed. In this manner, OVA-exposed mice and their corresponding (time-matched) saline controls were assayed on the same day. All animals and samples were assayed by the same individual.

Bronchoalveolar Lavage (BAL) Assays

Immediately after the assessment of airway reactivity, lungs were lavaged via a tracheal cannula with Hanks balanced salt solution. Total leukocyte numbers were measured with a Coulter Counter (Coulter Corporation, Hialeah, FL). Differential cell counts were made from cytocentrifuged preparations using a Cytospin 2 (Shandon Ltd., Runcorn, Cheshire, UK) after staining with Leukostat (Fisher Diagnostics, Pittsburgh, PA). All slides were inspected by the same individual in a blinded manner. Cells were identified by standard morphology and differentiated into neutrophils, eosinophils, epithelial cells, lymphocytes, and macrophages. For each slide, at least 200 cells were counted under $\times 1000$ magnification.

Bronchoalveolar lavage (BAL) supernatants were collected and kept frozen at -80°C until assayed. The levels of macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), substance P and cysteinyl leukotrienes (CL) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. Kits for MIP-2, KC, and CL were from R&D Systems (Minneapolis, MN), whereas the substance P ELISA kit was from Assay Designs (Ann Arbor, MI). The detection limits were 1.5, 2, 27, and 8 pg/ml for MIP-2, KC, CL, and substance P, respectively. Values below the detection limit were set at zero.

Lung Histopathology and Morphometric Analyses

The lungs were fixed *in situ* by injection of 1 ml 10% buffered formalin solution directly into the lungs using a cannula fixed by a ligature around the trachea. The lungs were subsequently removed and placed in fixative and processed into paraffin. Mucus-containing goblet cells were detected by staining of paraffin sections

(5- μm thick) with periodic acid-Schiff (PAS). Goblet-cell metaplasia was evaluated by quantification of the number of PAS+ stained cells per millimetre surface epithelium in bronchi and bronchioles. Furthermore, the amount of stored mucosubstances was assessed by calculating the ratio of PAS+ stained area to total investigated area. The morphometric analyses were performed using a light microscope attached to a computer using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD). Data were exported to Microsoft Excel for further analysis.

Statistical Analyses

Data from the airway response to MCh exposures, BAL, and histological evaluation are presented as means $\pm$ SEM for pooled data from two independent experiments each with four mice per group (Figures 1–3 and Table 1). The reactivity to MCh (Figure 1) was assessed by comparing peak responses in the OVA-exposed mice with the time-matched saline control group by the Mann-Whitney *U*-test.

In order to minimize the number of statistical tests and thereby reduce the risk of type I errors, the following procedure was followed for the analyses of BAL cell numbers (Figure 2), histology (Figure 3), and levels of CL and KC (Table 1). Initially, data were analyzed by means of two-way analysis of variance (ANOVA) comparing ozone concentration (0 (air), 100, 250, or 500 ppb) and 10-d exposures (OVA, saline). Two separate ANOVA tests were performed: one for data obtained 6 h after and another for data obtained 24 h after ozone exposure. If ANOVA showed a treatment-dependent effect ($p < .05$), data were further analyzed using two-sample *t*-tests. In the *t*-tests, OVA-challenged mice were compared to saline-challenged control mice exposed to the same concentrations of ozone (referred to as the corresponding control group).

Statistical analyses were performed using the Minitab statistical analysis program, release 15 (Minitab, Inc., State College, PA). Significance was considered at *p* values less than .05. Figure 1 was constructed using a Microsoft Excel curve smoothing function.

TABLE 1. Effects of OVA and ozone Exposures on Levels of Inflammatory Mediators in BALF

Time of BALF recovery	10-day exposure	3-hr exposure on day 11	CL (pg/ml)	KC ^a (pg/ml)	MIP-2 ^a (pg/ml)
6 hr	Saline	Air	1180 ± 134	16 ± 2.9	0.14 ± 0.12
		100 ppb O ₃	736 ± 171	11 ± 3.5	0.28 ± 0.26
		250 ppb O ₃	164 ± 30	13 ± 2.6	1.37 ± 0.55
		500 ppb O ₃	962 ± 282	99 ± 15	12.2 ± 2.6
	OVA	Air	541 ± 128	15 ± 3.5	1.07 ± 0.41
		100 ppb O ₃	627 ± 198	15 ± 1.8	0.47 ± 0.43
		250 ppb O ₃	213 ± 56	13 ± 3.3	0.56 ± 0.37
		500 ppb O ₃	807 ± 193	51 ± 4.0*	3.6 ± 0.6*
24 hr	Saline	Air	827 ± 216	15 ± 3.7	0.91 ± 0.71
		100 ppb O ₃	919 ± 274	5.3 ± 0.9	0 ± 0
		250 ppb O ₃	557 ± 202	7.3 ± 2.7	1.0 ± 0.82
		500 ppb O ₃	1255 ± 230	15 ± 3.2	0.27 ± 0.16
	OVA	Air	701 ± 164	13 ± 1.8	0.22 ± 0.14
		100 ppb O ₃	310 ± 42	6.3 ± 1.4	0.11 ± 0.11
		250 ppb O ₃	319 ± 65	7.2 ± 1.6	0.76 ± 0.40
		500 ppb O ₃	838 ± 267	12 ± 1.6	0.24 ± 0.16

Values are means ± SEM for groups of 8 mice. Mice were exposed for 10 consecutive days either to saline or a 1% OVA aerosol. On day 11, mice were exposed 3 hours to air, 100, 250, or 500 ppb ozone, respectively. Levels of cysteinyl leukotrienes (CL), macrophage inflammatory protein 2 (MIP-2), and keratinocyte-derived chemokine (KC) in bronchoalveolar lavage fluid (BALF) 6 or 24 hours after end of the 3-hour exposure period were measured by ELISA, cf. the Method section. Statistically significant differences between an OVA group and its time-matched saline control group (cf. Method section) is indicated by * ($p < 0.05$).

^aSamples with a chemokine level below the limit of detection (cf. Method section) were set to zero.

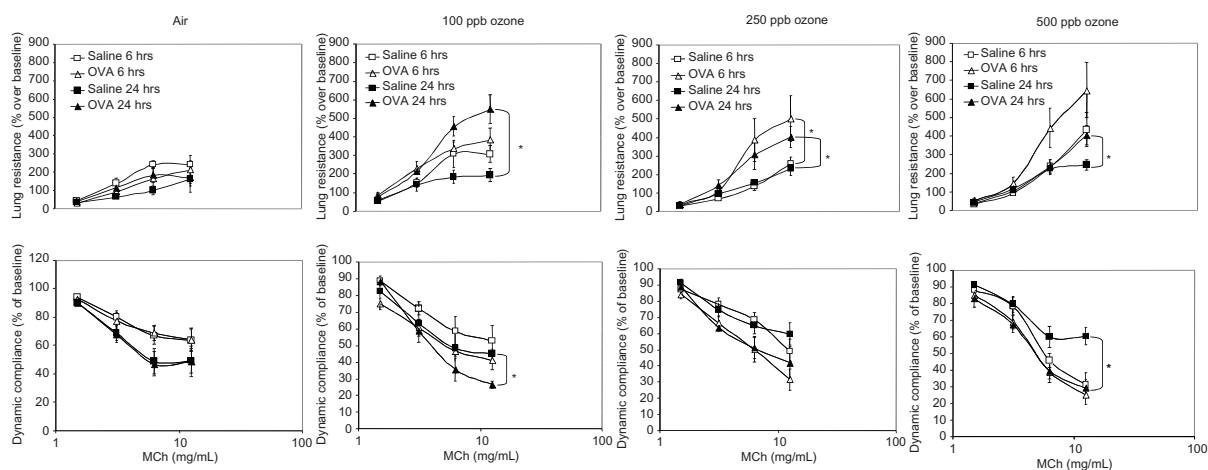


FIGURE 1. Airway resistance and dynamic compliance to inhaled methacholine. Data are shown as means ± SEM for groups of eight mice. Mice were exposed for 10 consecutive days to aerosols of either saline or a 1% OVA solution followed by a 3-h exposure to air or ozone. Asterisk indicates significant difference ($p < .05$) between indicated groups.

RESULTS

Airway Reactivity

In the studies examining airway responsiveness, two conditions were important to meet. The first was to demonstrate that the

exposure of mice on 10 consecutive days to OVA followed by 3-h air challenge did not elicit AHR to inhaled MCh. As shown in Figure 1, exposure to air did not result in increased MCh reactivity in mice that were exposed to either OVA or saline over 10

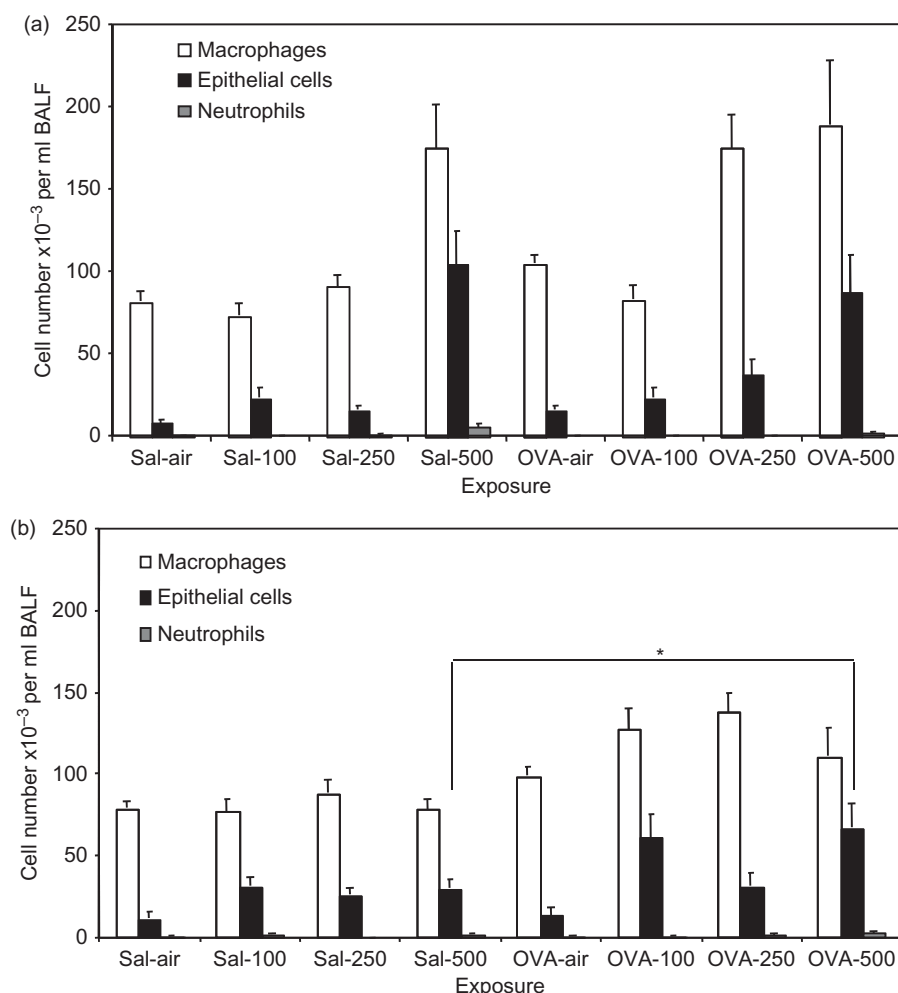


FIGURE 2. Number of cells in BAL fluid. Mice were exposed for 10 consecutive days to aerosols of either saline (Sal) or a 1% OVA solution followed by a 3-h exposure to air or ozone (concentration given in ppb). A statistically significant difference between an OVA-exposed group and the saline-exposed control group receiving the same concentration of ozone is indicated by an asterisk, significant at $p < .05$, at (a) 6 h after ozone exposure or (b) 24 h after ozone exposure. Data are means $\pm$ SEM for groups of eight mice.

consecutive days. The second variable was to establish whether ozone exposure by itself could elicit AHR independent of OVA inhalation. As shown in Figure 1, the response to ozone (in the absence of OVA exposure) was marginal at both 6 and 24 h postexposure at all 3 levels of ozone. Prior 10-d OVA exposure enhanced the response to ozone at all 3 levels of exposure: The maximal changes following exposure to 100 ppb were at 24 h; changes were detected at 6 and 24 h exposure to 250 ppb ozone; and 500 ppb ozone elicited increased AHR only 24 h after ozone

exposure. In mice exposed to 100 or 500 ppb ozone, the increases in lung resistance were accompanied by decreases in dynamic compliance (Figure 1).

Bronchoalveolar Lavage

Data showed that 10-d exposure to OVA did not markedly alter the number of macrophages compared to 10-d saline exposure at 6 or 24 h after ozone exposure (Figure 2). Furthermore, results demonstrated that there was no effect of OVA exposure on the number of epithelial cells 6 h after ozone exposure.

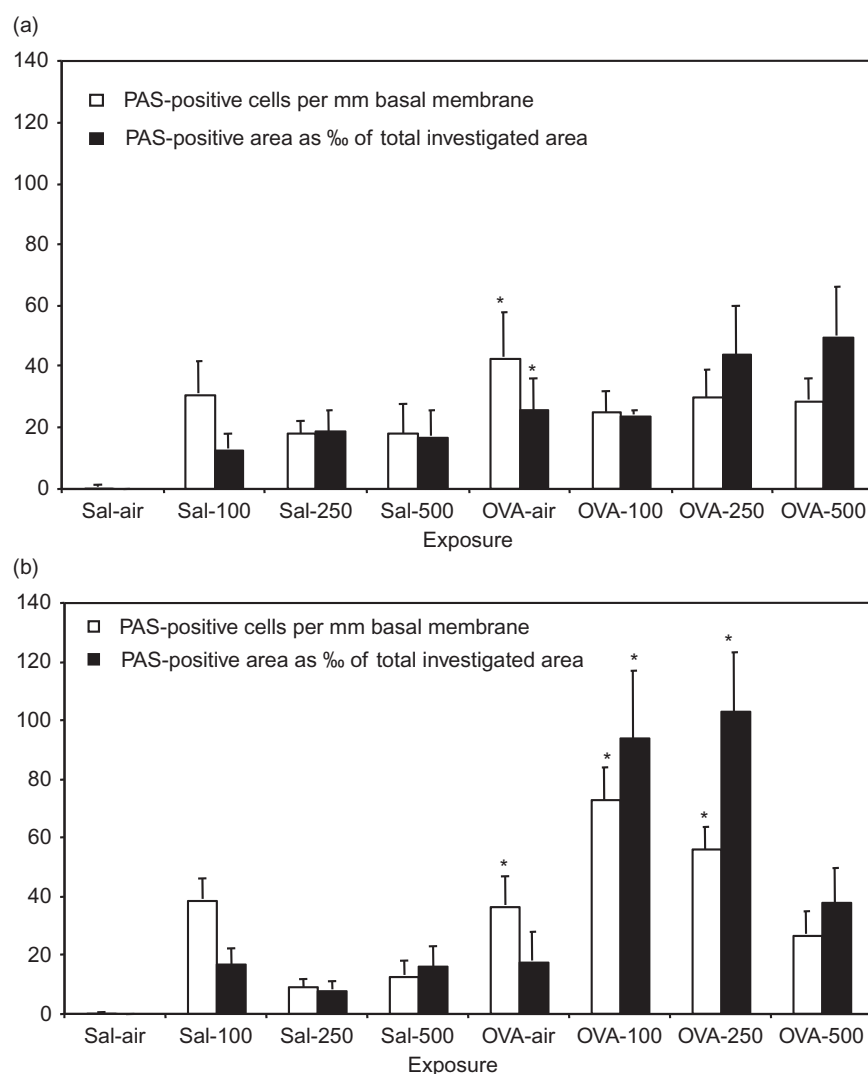


FIGURE 3. Numbers of PAS+ cells per millimetre basal membrane and amount of stored mucus in goblet cells assessed by histology. Mice were exposed for 10 consecutive days to aerosols of either saline (Sal) or a 1% OVA solution followed by a 3-h exposure to air or ozone (concentration given in ppb). An increased number of PAS+ cells or an increased amount of stored mucus when compared to the saline-exposed control group receiving the same concentration of ozone is indicated by an asterisk, significant at $p < .05$, at (a) 6 h after ozone exposure or (b) 24 h after ozone exposure. Data are means $\pm$ SEM for groups of eight mice.

However, 24 h after ozone exposure, a significantly higher number of epithelial cells was seen in the OVA-500 ppb group compared to the Sal-500 group, indicating that OVA exposure prior to ozone may further enhance ozone-induced epithelial desquamation and damage.

The numbers of neutrophils were low in all groups, although the number tended to be elevated in mice exposed to 500 ppb ozone (Figure 2, a and b).

Levels of MIP-2 and KC were low at 0, 100, and 250 ppb ozone exposure after 6 and 24 h, regardless of exposure to OVA (or saline), but levels of both MIP-2 and KC increased after exposure to 500 ppb ozone. Six hours after exposure to 500 ppb ozone, significantly lower levels of MIP-2 and KC were seen in mice exposed to OVA when compared to saline-exposed mice. No exposure-dependent effects were seen with CL (Table 1). All substance P levels were below the limit of detection (data not shown).

Histological Examination

Goblet-cell metaplasia was assessed by quantifying the number of PAS+ cells per millimetre basal membrane, and the mucus-producing activity of the goblet cells was determined by measuring stored mucosubstances (PAS+ area as percent of total investigated area). Ten-day exposure to OVA followed by 3-h air challenge gave rise to increases in numbers of goblet cells compared to mice exposed for 10 d to saline followed by 3 h of air (Figures 3a and 3b). Exposure to 10 d of OVA followed by 3-h exposure to 100 or 250 ppb ozone produced significantly elevated numbers of PAS+ cells and PAS+ stained areas, 24 h after ozone

exposure, compared to saline control animals exposed to the same concentrations of ozone (Figure 3b). No marked differences were seen between the Sal-500 and OVA-500 groups at 6 or 24 h postexposure. Histology revealed that the 500-ppb ozone level may be toxic to the mucus-containing cells, and vacuolization was observed in these cells (Figure 4).

DISCUSSION AND CONCLUSIONS

Mouse models are extensively used in order to better understand the mechanisms underlying the development of asthma. Numerous such models have been developed, using various

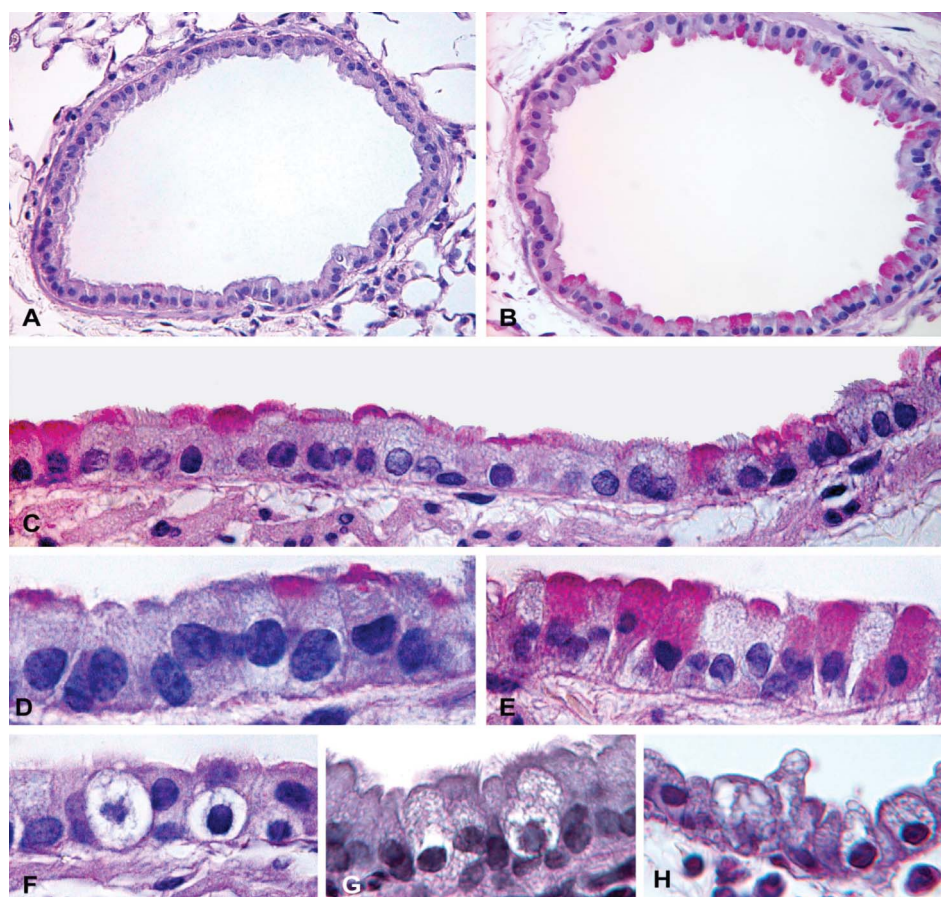


FIGURE 4. Bronchioles from (A) a control and (B) an ozone-exposed mouse stained with PAS to demonstrate the presence of mucus (red) in the latter whereas the normal epithelium is without mucus in any cells. (C) Segment of epithelium from bronchiole showing ciliated cells and Clara cells with varying amounts of mucus. (D) Larger magnification showing mucus as a luminal rim in Clara cells. (E) Clara cells having a goblet-cell-like appearance. (F), (G), and (H) illustrate varying degrees of Clara-cell vacuolization or degeneration in mice exposed to 500 ppb ozone. It is apparent especially in (G) that the ciliated cells have a normal appearance. Magnifications: A, B: $\times 125$, C: $\times 380$, D: $\times 750$, E: $\times 600$, F: $\times 500$, G: $\times 600$; and H: $\times 500$.

mouse strains, antigen doses, routes of antigen administration, or numbers of antigen challenges, as recently reviewed (Kumar et al., 2008). Usually, models utilizing the intraperitoneal route for sensitization followed by airway challenge induce high numbers of eosinophils in BAL. However, eosinophils typically account for only 1–3% of cells in by BAL from human asthmatics, whereas in these mouse models there are major differences, including the accumulation of high number of eosinophils in the airways (Fulkerson et al., 2005). In mice, from exposure to aerosolized allergen via the airways induces lower numbers of eosinophils, as demonstrated in the present study. Thus, protocols based on inhalation of allergen exclusively more closely resemble the exposure to allergens seen in patients with asthma.

The present study demonstrated that prior exposure to aerosolized allergen on 10 consecutive days increases AHR to ozone as evidenced by increases in lung resistance and decreases in dynamic compliance. This additive effect was seen at all ozone concentrations, including 100, 250, and 500 ppb. At 100 ppb ozone, a concentration that can be experienced in urban and occupational environments, the heightened airway reactivity was most prominent 24 h after exposure to ozone. These data support the findings in clinical studies, where an association between ozone exposure and the number of emergency visits for asthma were detected the following day (lag 1 d) (Romieu et al., 1995). Furthermore, prior exposure to OVA may lead to a more slowly resolving AHR in ozone-exposed mice, most clearly seen at 100 ppb where the combination of OVA and ozone exposure resulted in AHR that was maintained 24 h after ozone exposure. OVA-exposed animals developed AHR 6 h after exposure to 250 ppb ozone, suggesting that the 250 ppb ozone was sufficient to trigger an earlier AHR response compared to 100 ppb. Mice pre-exposed to OVA responded more vigorously to subsequent ozone exposure both at the 100- and 250-ppb levels. This is in contrast to 500 ppb, where saline-exposed control mice also were affected 6 h after ozone exposure. OVA-exposed mice had, however, AHR

24 h after 500 ppb ozone exposure, suggesting a more persistent response in these animals. Thus at high levels of ozone, all mice were affected by the exposure, an observation that is in agreement with previous studies demonstrating that ozone exposure can increase airway resistance in nonsensitized animals (Watanabe et al., 1973) and decrease dynamic compliance (Gordon & Amdur, 1980).

Yamauchi et al. (2002) studied the acute effects of ozone exposure on lung function in mice sensitized to OVA. Mice exposed to the combination of OVA and ozone showed decreased dynamic compliance more than mice exposed to OVA alone. However, in contrast to the present study, Yamauchi and coworkers (2002) did not see a significant effect on lung resistance to inhaled MCh. This may be due to differences in the immunization protocol, as Yamauchi and coworkers (2002) immunized with OVA and alum using the ip route followed by nebulization of OVA, whereas 10 daily OVA aerosol exposures was used in the absence of adjuvant in this study. Further, they used C57BL/6 mice, which are known to differ significantly in their responses to OVA, especially in response to inhaled MCh (Takeda et al., 1997).

Ten-day exposure to OVA produced an increase in numbers of goblet cells, but the highest degree of goblet-cell metaplasia was seen when OVA-exposed mice were subsequently exposed to ozone. This observation is in agreement with observations in respiratory epithelium from Brown Norway rats exposed either to OVA alone, ozone alone, or a combination of OVA and ozone (Wagner et al., 2002). The increases in goblet-cell metaplasia were observed 24 h, but not 6 h, after exposure to 100 or 250 ppb ozone. It may be that ozone triggered release of mucin from the goblet cells, explaining the lower levels of stored mucin 6 h after ozone exposure. At 24 h after ozone exposure, the cells regenerated their mucin content. Exposure to OVA and 500 ppb ozone did not lead to goblet-cell metaplasia, most likely due to a toxic effect of ozone on the goblet cells at higher concentrations. A toxic effect of 500 ppb ozone on the mucus-producing cells was

confirmed by histology. Furthermore, the highest ozone concentration (500 ppb) produced epithelial-cell desquamation, which is a well-established effect of ozone (Brunekreef & Holgate, 2002). Respiratory epithelial cells from rats repeatedly exposed to ozone can adapt, minimizing the injury to the epithelium over time (Tesfaigzi, 2003). Humans in general are believed to be more sensitive to ozone exposure than animals (Bhalla, 1999).

The CXC chemokines MIP-2 and KC are known to be important chemotactic mediators involved in neutrophil recruitment (Holgate, 2005). The highest levels of MIP-2 and KC were seen 6 h after exposure to 500 ppb ozone. In addition, these groups had the highest number of neutrophils in BAL fluid, although the increased cell numbers did not reach statistical significance. Mice exposed for 10 d to OVA had a lower KC and MIP-2 response 6 h after exposure to 500 ppb ozone when compared to the saline control animals. As mucus contains antioxidants such as ascorbate, vitamin E, and urate, the increased mucus layer in the OVA-exposed animals may provide some degree of protection against the acute oxidative effects of ozone.

Airway exposure to endotoxin, including lipopolysaccharide, often results in increased numbers of neutrophils in BAL fluid (Moon et al., 2009). Purified OVA, which was used in the present study, may still contain trace amounts of endotoxin (below the detection limit in the assays), but the low number of BAL neutrophils observed in the present study suggests that the level of endotoxin in the OVA preparations was not sufficient to recruit neutrophils to the lungs.

No apparent eosinophilic lung inflammation was seen after the 10-d exposure to OVA aerosol. Similarly, mice exposed to OVA by inhalation in the absence of adjuvant and without preceding parenteral sensitization did not develop eosinophilic airway inflammation (Larsen et al., 2007; Siegle et al., 2006). Thus, in our study, ozone-induced AHR was independent of eosinophilic inflammation, which is consistent with other studies of the role of eosinophils in AHR (Siegle et al., 2006). Yost et al. (1999) proposed that AHR induced by ozone in sensitized guinea pigs may be due to the release of major basic

protein from activated eosinophils in the airways. The absence of eosinophilic inflammation in the present model indicates that in mice, an alternative mechanism may be involved.

The present study showed that OVA-exposed mice developed AHR after a single 3-h exposure to 100 ppb ozone, a level often exceeded during urban smog episodes. AHR developed slowly and was significant 24 h after ozone exposure was completed. This is in keeping with an epidemiological study showing that a smog episode leads to increased asthma hospital admissions after a 1-d lag period (Romieu et al., 1995). The development of AHR was associated with goblet-cell metaplasia and greater mucus production but not with airway eosinophilia, distinguishing it from allergen-induced AHR.

In conclusion, our study showed a clear interaction between ozone exposure and allergen exposure. In mice exposed to allergen, even low levels of ozone (100–250 ppb) may lead to AHR and goblet-cell metaplasia. At higher ozone concentrations (500 ppb), mechanisms more related to the toxicity of ozone may also play a role. Given the combined effects of allergen and ozone, it is important to determine whether the current environmental and industrial “low-level” exposure guidelines can be considered safe, especially for those with established airway disease and asthma.

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