Enhancement of Nasal Inflammatory and Epithelial Responses after Ozone and Allergen Coexposure in Brown Norway Rats

James G. Wagner, Jon A. Hotchkiss, and Jack R. Harkema

Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, Michigan 48824

Received October 5, 2001; accepted January 25, 2002

Repeated exposures to ozone cause inflammation and mucous cell metaplasia (MCM) in the nasal mucosa of laboratory animals. Similar cellular responses occur in humans during allergic rhinitis. We tested the hypothesis that exposure to ozone will enhance the inflammatory and epithelial responses associated with allergic rhinitis. Ovalbumin (OVA)-sensitized Brown Norway rats were exposed to ozone (0.5 ppm, 8 h/day) for 1 day or 3 consecutive days. Immediately after each ozone exposure, animals were challenged intranasally (IN) with either sterile saline or OVA dissolved in saline (1%, 50 μl/nasal passage). Twenty-four h after the last IN challenge rats were sacrificed; nasal tissues were removed and processed for light microscopic examination and morphometric analysis of numeric densities of inflammatory and epithelial cell populations and volume densities of intraepithelial mucosubstances. A single OVA challenge caused a significant influx of neutrophils and eosinophils into the submucosa of all nasal tissues. Ozone exposure further enhanced the appearance of eosinophils in the maxilloturbinate of OVA-challenged rats but did not increase inflammation in other nasal tissues. After 3 days of ozone/OVA coexposures, the nasal transitional epithelium lining the maxilloturbinate had increased numbers of epithelial cells as well as the appearance of mucus-containing cells in areas normally absent of these secretory cells (i.e., MCM). Multiple challenges with OVA caused increased epithelial mucosubstances in the respiratory epithelium lining the septum without increasing the number of epithelial cells. Multiple exposures to both ozone and OVA caused greater increases in intraepithelial mucosubstances in the septum than those elicited by OVA alone. These results demonstrate that exposure to ozone exacerbates epithelial and inflammatory responses associated with allergen challenge. In addition, coexposure of these agents enhanced the induced production of nasal mucosubstances caused by either agent alone.

Key Words: ozone; allergen; ovalbumin; mucous cell metaplasia; eosinophil; neutrophil; inflammation.

Exposure of humans to ambient ozone, the primary oxidant pollutant found in photochemical smog, causes inflammation and histological alterations in the mucosal surface of nasal passages (Calderon-Garciduenas, et al., 1994; Frischer et al., 1993). Histopathologic changes in people include a neutrophilic rhinitis, increased mucus secretion, epithelial cell injury and hyperplasia/metaplasia. We have documented similar ozone-induced responses in rats, as well as described the development of epithelial cell hyperplasia and mucous cell metaplasia (MCM) after acute and chronic ozone exposures (Harkema et al., 1989, 1997; Hotchkiss et al., 1998).

Among major air pollutants, ozone is currently considered one of the most pervasive problems in regard to the attainment of the National Ambient Air Quality Standards (NAAQS). The U.S. Environmental Protection Agency (U.S. EPA) estimates that almost 50% of the United States population lives in areas where ambient ozone levels exceed NAAQS limits (U.S. EPA, 2000). Approaches to establishing safe ozone exposure limits for humans involve consideration for the most susceptible populations. In this regard, people with preexisting respiratory conditions such as asthma and chronic obstructive pulmonary diseases are considered at risk for adverse health effects from exposure to high ambient ozone concentrations that occur during summer months (Bascom et al., 1995; Cody et al., 1992; White et al., 1994). Results from both human and animal studies show that ozone exacerbates airway hyperresponsiveness and immune and inflammatory responses in lower airways of asthmatic subjects (Gilmore, 1995; Hiltermann et al., 1998; Jenkins et al., 1999; Vagaggini et al., 1999).

Rhinitis is frequently associated with asthma, and similar mechanisms are postulated to underlie the inflammatory and physiologic responses of each condition (Casale, 1999; Simmons, 1999; Vignola et al., 1998). As such, upper airway responses to ozone might be exacerbated in individuals with allergic rhinitis by mechanisms similar to those that occur in lower airways of asthmatics after ozone exposure. Both healthy and asthmatic individuals respond to ozone exposure with nasal inflammation as indicated by the presence of neutrophils, eosinophils, and inflammatory mediators in nasal lavage fluid (Graham and Koren, 1990; Hiltermann et al., 1997). However, these lavage markers of ozone-induced inflammation are greater in asthmatics than in nonasthmatics (McBride et al., 1994). Furthermore, ozone enhances some antigen-specific allergic nasal responses. Concentrations of inflammatory cells...
and soluble mediators in nasal lavage are increased after allergen challenge when allergic subjects are first exposed to ozone (Bascom et al., 1990; Peden et al., 1995). Thus, in addition to the sensitive populations mentioned above (i.e., asthmatics, children, etc.), people with allergic rhinitis might also be included as a susceptible target group when assessing the risks to human health of ozone exposure. Indeed, recent epidemiological studies in the United Kingdom demonstrate a 37% increase in allergic rhinitis patients on days following high ambient concentrations of ozone (Hajat et al., 2001).

The mechanism or mechanisms by which ozone enhances allergic inflammatory responses in the nasal airways of humans are unknown. Similar studies in laboratory animals have not been performed. Apart from inflammatory cell infiltration, alteration in nasal epithelial cells, and the production and secretion of mucus are common symptoms in humans with allergic rhinitis (Varney et al., 1992; Watanabe and Kiuna, 1998). Although allergic asthma has been well characterized in animal models, there are few animal models of allergic rhinitis that describe the alterations in epithelial and mucous cells (Shimizu et al., 2000). Conversely, we have developed sensitive techniques to examine the inflammatory, histologic, and morphologic changes in rat nasal epithelium after acute and chronic exposures to ozone (Harkema et al., 1989, 1997).

Because ozone exacerbates responses to allergens in the lower airway and promotes mucous cell changes by itself in upper airways, we hypothesized that exposure to ozone will enhance nasal epithelial responses in a rat model of allergic rhinitis. In the present study we exposed sensitized rats to ozone and allergen, and used histochemical, image analysis, and morphometric techniques to characterize the development of inflammation and morphologic alterations in the nasal epithelium. Specifically, we examined two types of nasal epithelium that line specific sites in proximal nasal airways: the respiratory epithelium (RE), a pseudostratified, ciliated epithelium that lines the mid-nasal septum and contains several mucous (goblet) cells, and the nasal transitional epithelium (NTE), a nonciliated, cuboidal epithelium that lines the maxilloturbinates and contains few, if any, mucous cells (Harkema et al., 1991). In general, allergen elicits mucous cell responses in the RE (Varney et al., 1992), and ozone causes mucous cell metaplasia in the NTE (Cho et al., 1999, 2000; Harkema et al., 1989; Hotchkiss et al., 1997). In this manner, we were able to compare the responses of distinct nasal tissues to these agents and determine the potential interactions of ozone- and allergen-engendered responses. Using morphometry and histologic descriptions of epithelial cell remodeling, we extended the findings of human studies that used nasal lavage to indicate ozone enhancement of allergic rhinitis. Moreover, our results provide the first evidence in animals or humans that ozone exposure enhances the nasal inflammatory and epithelial alterations caused by exposure to allergens.

MATERIALS AND METHODS

Animals. Forty-eight male Brown Norway rats (Charles River, Portage, MI), 10–12 weeks of age, were randomly assigned to one of eight experimental groups (n = 6). Animals were housed individually in rack-mounted, stainless steel wire cages in whole-body inhalation exposure chambers (HC-I 00, Lab Products, Maywood, NJ), with free access to tap water and food (Tek Lad 1640, Harlan Sprague-Dawley, Indianapolis, IN). The chamber temperature and relative humidity were maintained between 21–24°C and 40–55%, respectively. Room lights were set on a 12-h light/dark cycle beginning at 6:00 A.M.

Ozone exposure. Rats were exposed to filtered air or 0.5 ppm ozone for either 1 or 3 days for 8 h/day. The ozone concentration used produces minimal lesions in the NTE of Brown Norway rats in acute studies, and has little or no effect on respiratory epithelium (Harkema et al., 1999). By comparison, ozone induces a pronounced MCM in the NTE of F344/N rats, but these rats are not highly responsive to allergen sensitization and intranasal challenge (Harkema et al., 1989, 1997; Hotchkiss et al., 1999). Thus, the Brown Norway rat provides a good animal model of allergic rhinitis in which to test the effects of ozone exposure.

Ozone was generated with an OREC model 03VI-I ozonizer (Ozone Research and Equipment Corp., Phoenix, AZ) using compressed air as a source of oxygen. Total airflow through the exposure chambers was 250 l/min (15 chamber air changes/h). The concentration of ozone within chambers was monitored throughout the exposure using two Dasibi 1003 AH ambient air ozone monitors (Dasibi Environmental Corp., Glendale, CA). Sampling probes were placed in the breathing zone of rats within the middle cage racks. The concentration of ozone during exposures was 0.536 ± 0.008 ppm (mean ± SEM) for ozone chambers and less than 0.02 ppm for chambers receiving filtered air.

Allergen exposure. Immediately after each inhalation exposure, rats were removed from the chambers, anesthetized with 4% halothane in oxygen, and 50 μl of either pyrogen-free saline or an ovalbumin solution (1%, prepared in pyrogen-free saline) was instilled into each nasal airway passage of rats. Rats were exposed to ozone for 8 h to elicit inflammatory cell infiltration, which would be present at the time of intranasal ovalbumin instillation. Animals were returned to the inhalation chambers after IN challenges.

Necropsy and tissue preparation. Twenty-four rats were killed at 24 h after a single inhalation and intranasal challenge, and twenty-four were killed 24 h after three daily inhalation and intranasal treatments. Two h before necropsies, rats were injected intraperitoneally with bromodeoxyuridine (BrdU; 50 mg/kg body weight) to label epithelial cells undergoing DNA synthesis (s-phase of cell cycle). Rats were killed via exsanguination by cutting the abdominal aorta, after being deeply anesthetized with sodium pentobarbital (50 mg/kg, ip). Immediately after death, the head of each animal was removed from the carcass. After the lower jaw and skin were removed, the head was placed in a large volume of zinc-formalin (Anatech, Kalamazoo, MI) for at least 48 h. After fixation, the head was decalcified in 13% formic acid for 4 days, and then rinsed in tap distilled water for 4 h. A tissue block was removed from the anterior nasal cavity by making two cuts perpendicular to the hard palate: (1) immediately posterior to the upper incisors, and (2) at the level of the incisive papilla (Fig. 1A). The tissue blocks were embedded in paraffin, and 5 to 6 μm-thick sections were cut from the anterior surface. Nasal sections were stained with hematoxylin and eosin (H&E) for routine histology, Alcian blue (pH 2.5)/periodic acid-Schiff AB/PAS) to detect intraepithelial mucousubstances, May-Grünewald stain and hematoxylin to detect eosinophils, or immunohistochemically to detect BrdU-labeled cells. Neutrophils were identified by morphologic characteristics that include their size, their darkly stained, multinucleated nuclei, and their clear cytoplasm with dust-like granules. In contrast, eosinophils were slightly larger in size than neutrophils, had a bilobed nucleus, and contained numerous intracytoplasmic eosinophilic granules.

Immunocytochemistry. Hydrated paraffin sections (5–6 μm thick) from formalin-fixed nasal tissues were treated with 0.05% proteinase K for 2 min
and washed with 1 N HCl for 1 h. Sections were then treated with 3% H2O2 (in methanol) to block endogenous peroxidase and were incubated with a monoclonal antibody to BrdU (Accurate Chemical and Scientific Corp., Westbury, NY) for 1 h at a dilution of 1:20. Immunoreactive BrdU was visualized with the Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) using 3,3′-diaminobenzidine (DAB) tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) as a chromagen.

**FIG. 1.** Locations of nasal tissues selected for analyses. (A) Exposed lateral wall of nasal airway. The vertical lines indicate the anterior surface of each transverse block used for morphometric study. N, nares; NT, nasoturbinate; MT, maxilloturbinate; ET, ethmoids; HP, hard palate; b, brain; and NP, nasopharynx. (B) Anterior face of tissue block from (1) proximal and (2) distal nasal airways: The areas of mid-septum and maxilloturbinates in nasal section 1 (proximal airway) that were used for morphometric analyses are within the boxes. S, septum; HP, hard palate; m, maxilloturbinate; n, nasoturbinate; lw, lateral wall; vmm, ventral medial meatus; dmm, dorsal medial meatus; and vlm, ventral lateral meatus.

and washed with 1 N HCl for 1 h. Sections were then treated with 3% H2O2 (in methanol) to block endogenous peroxidase and were incubated with a monoclonal antibody to BrdU (Accurate Chemical and Scientific Corp., Westbury, NY) for 1 h at a dilution of 1:20. Immunoreactive BrdU was visualized with the Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) using 3,3′-diaminobenzidine (DAB) tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) as a chromagen.

**Morphometry of stored intraepithelial mucosubstances.** To estimate the amount of the intraepithelial mucosubstances in NTE and RE, the volume density (Vs) of AB/PAS-stained mucosubstances was quantified using computerized image analysis and standard morphometric techniques. The area of AB/PAS-stained mucosubstances was calculated from the automatically circumscribed perimeter of stained material on a Power Macintosh 7100/66 computer using the public domain NIH Image program (written by Wayne Rasband, U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances per unit length of surface area of epithelial basal lamina was determined as described previously (Harkema et al., 1987). It was expressed as nl of intraepithelial mucosubstances per mm² of basal lamina (i.e., volume density).

**Morphometry of epithelial DNA synthesis and cell density.** The number of BrdU-labeled nuclei divided by the total epithelial nuclei × 100 (percentage of BrdU-labeled epithelial cells) was used as an estimate of DNA synthesis in the NTE and RE. The numeric epithelial cell density was determined by counting the number of epithelial cell nuclear profiles in the surface epithelium and dividing by the length of the underlying basal lamina. The length of the basal lamina was calculated from its contour length in a digitized image using the NIH image system described above.

**Morphometry of inflammatory cell densities.** The effect of ozone and ovalbumin exposures on neutrophil and eosinophil influx was determined in May-Grunwald stained sections by counting the total number of each cell type within the mucosa of the septum (area between the airway and septal cartilage) or mucosa of the maxilloturbinate (area between the airway and the turbinate bone). Granulocytes with a bilobed nucleus and large bright pink staining granular cytoplasm were counted as eosinophils, whereas smaller polymorphonuclear cells with clear cytoplasm that excluded May-Grunwald stain were identified as neutrophils.

**Statistical analysis.** Morphometric data are expressed as the mean ± the standard error of the mean (SEM) and were statistically analyzed using a completely randomized analysis of variance. Multiple comparisons were made by Student-Newman Keuls post hoc test. Criterion for significance was taken to be p < 0.05.

**RESULTS**

**Histopathology**

**Rats intranasally exposed to ovalbumin and filtered air.** Rats exposed to only one intranasal instillation of ovalbumin had a moderate to marked allergic rhinitis. This nasal response was characterized by a mixed inflammatory cell infiltrate of eosinophils, lymphocytes, plasma cells, and occasional neutrophils in the nasal mucosa lining most of the intranasal airways in both the proximal and distal nasal passages (Fig. 2B). This conspicuous eosinophilic and mononuclear inflammatory cell response was, however, restricted to the nasal mucosa containing ciliated, pseudostratified respiratory epithelium (RE) lining the proximal and distal nasal septum and the distal lateral meatus, and to mucosa containing nonciliated cuboidal epithelium (nasal transitional epithelium; NTE) lining the proximal lateral meatus. Interestingly, this inflammatory cell response was minimal or absent in the mucosa containing olfactory epithelium lining the dorsal medial meatus in the distal nasal section and in the mucosa containing squamous epithelium (SE) lining parts of the ventral middle meatus (proximal nasal airways only). Though there was infiltration of inflammatory cells in both the RE and NTE, there was no morphologic evidence of epithelial injury.

Animals that received 3 consecutive days of intranasal instillations to ovalbumin and filtered air had a more severe allergic rhinitis response than those rats that received only one intranasal instillation of ovalbumin (Figs. 2C and 3C). Though the cellular character and distribution of the allergen-induced nasal inflammation were similar in both groups of animals, there were greater numbers of inflammatory cells in the nasal mucosa of rats receiving the multiple instillations. In addition, these rats had conspicuous surface epithelial alterations con-
sisting of marked mucous cell hyperplasia and mucous cell metaplasia (MCM) in the RE lining the nasal septum in the proximal and distal nasal passages, respectively (Fig. 4).

Rats intranasally exposed to saline and ozone. Rats exposed to only a single intranasal instillation of saline and one 8-h inhalation exposure to ozone had a moderate to marked rhinitis that was restricted to the mucosal tissues containing NTE or RE, lining the proximal and distal lateral meatus, respectively. The inflammatory cell influx was composed mainly of eosinophils with considerably fewer numbers of neutrophils and mononuclear cells. Associated with this rhinitis was mild to moderate epithelial degeneration and necrosis of the NTE and RE lining the lateral meatus. Ciliated cells in the injured RE also had marked attenuation or loss of cilia. In the proximal nasal passage, these ozone-induced alterations were most pronounced in the NTE lining the lateral aspects of the nasal and maxilloturbinates and in the NTE on the lateral wall lining the middle lateral meatus. In the more distal nasal passage, the most conspicuous sites of epithelial injury were in the RE on the lateral wall lining the middle lateral meatus and on the most distal aspect of the maxilloturbinate.

FIG. 2. Light photomicrographs of the nasal mucosa lining the mid-septum from the proximal nasal airway of Brown Norway rats that received a daily intranasal challenge of saline without ovalbumin for 3 consecutive days (A, control), a single challenge of saline with 100 μg ovalbumin (B), or 100 μg ovalbumin in saline for 3 consecutive days (C). A mixed inflammatory cell infiltrate of mononuclear cells (e.g., lymphocytes, plasma cells) and eosinophils (arrows) are present in the subepithelial lamina propria and in the basal aspect of the respiratory epithelium (e) in (B) and (C). The respiratory epithelium (e) in (C) is markedly thickened compared to (A) and (B), and contains numerous mucous cells (m), with copious amounts of intracellular mucosubstances (mucous cell hyperplasia/hypertrophy); v, blood vessel in lamina propria; g, glands in lamina propria; sc, septal cartilage; and c, ciliated cell. Bar = 50 microns.

FIG. 3. Light photomicrographs of the distal end of the maxilloturbinate in the proximal nasal airways of ovalbumin-sensitized rats that were daily exposed for 3 consecutive days to (A) filtered air (0 ppm ozone) and an intranasal challenge of saline (no ovalbumin, control), (B) 0.5 ppm ozone and an intranasal challenge with saline, (C) filtered air (0 ppm ozone) and an intranasal challenge of ovalbumin in saline, or (D) 0.5 ppm ozone and an intranasal challenge of ovalbumin in saline. Ovalbumin-induced epithelial hyperplasia is present in the nonciliated surface epithelium (e, nasal transitional epithelium) of B (small arrows). Hyperplasia, along with mild, focal squamous cell metaplasia (small arrow) is evident in the transitional epithelium (e) in (D). Numerous eosinophils are present in the blood vessels and interstitium of the lamina propria (large arrows) in (C) and (D); tb, turbinate bone; v, blood vessel in lamina propria. Bars = 50 microns.
After three consecutive days of intranasal instillations of saline and 8-h inhalation exposures to ozone, the multiply exposed rats had a mild rhinitis with an associated regenerative hyperplasia of NTE and RE lining the lateral meatus in both the proximal and distal sections examined. The inflammatory cell influx consisted of eosinophils with lesser numbers of mononuclear cells (lymphocytes and plasma cells) and neutrophils. Though the intranasal distribution of the surface epithelial lesions was similar in the single and multiple exposures, the character of the epithelial lesions was markedly different.

The ozone-induced epithelial degeneration and necrosis observed after a single 8-h inhalation exposure was not a consistent feature in the nasal passages of the multiply exposed rats. Instead, the affected NTE and RE were distinctively hyperplastic, consisting of increased numbers of hypertrophic cells with basophilic cytoplasm and enlarged nuclei with prominent nucleoli (Fig. 3B). Attenuation and loss of cilia also was a characteristic feature of the regenerative RE lining the lateral wall in the distal nasal passage. RE lining the nasal septum was free of ozone-induced degeneration or necrosis, but there was a mild MCM in the proximal nasal passage.

**Rats exposed to both ovalbumin and ozone.** Rodents exposed to both a single intranasal instillation of ovalbumin and a single 8-h inhalation exposure to ozone had both ovalbumin- and ozone-related nasal lesions. These rats had a marked rhinitis characterized by a mixed inflammatory cell influx of numerous eosinophils and neutrophils with lesser numbers of mononuclear cells (lymphocytes and plasma cells). Like the other rodents that were exposed to either ozone or ovalbumin, the nasal inflammation was restricted to the nasal mucosa lined by NTE or RE and not in areas lined by olfactory or squamous epithelium. The rhinitis appeared to be most severe in the mucosa lining the lateral meatus in both the proximal and distal sections. The inflammation was also conspicuous in septal mucosa lining the middle meatus.

Associated with the rhinitis were prominent alterations in the NTE and RE of the affected mucosa. The NTE on the lateral wall, maxilloturbinates, and lateral surfaces of the nasoturbinate were markedly hyperplastic, with focal areas of mild to moderate MCM and nonkeratinizing squamous metaplasia (SM; Fig. 3D). The latter lesion was usually present on the dorsolateral surfaces of the maxilloturbinates and the lateral ridge and scroll of the nasoturbinate. Both the MCM and the SM were metaplastic lesions of the NTE that were not present in rats exposed only to ovalbumin or ozone.

The principal lesion of the RE lining the mid-septum in both the proximal and distal nasal passage was a marked MCM (Fig. 4). The RE lining the lateral wall in the distal nasal passage was markedly hyperplastic with attenuation and loss of cilia.

---

**FIG. 4.** Light photomicrographs of the nasal mucosa lining the mid-septum (A, B, C, D) and the mid-shaft of the maxilloturbinate (E, F, G, H) from the nasal airway of Brown Norway rats. Tissues were stained only with Alcian blue (pH 2.5)/periodic acid Schiff (AB/PAS) to identify neutral and acidic mucosubstances in the surface epithelium (e) lining the septum and the lateral and medial aspects of the maxilloturbinate. Rats were exposed for 3 consecutive days to filtered air (0 ppm ozone) and intranasal saline (A and E), 0.5 ppm ozone and intranasal saline (B and F), filtered air and ovalbumin in saline (C and G), or 0.5 ppm ozone and ovalbumin in saline (D and H). Increased amounts of AB/PAS-stained mucosubstances (arrows) are present in the surface epithelium lining the septum in (C) and (D) compared to (A) and (B). No AB/PAS-stained mucous cells are present in (E), (F), or (G). A few widely scattered AB/PAS-stained mucous cells (mucous cell metaplasia) are present in surface epithelium lining the lateral and medial aspects of the maxilloturbinate in (H); tb, turbinate bone; v, blood vessel in lamina propria; g, glands in lamina propria; SC, septal cartilage. Bars = 50 microns.
The epithelial alterations caused by ozone, ovalbumin, and the coexposure to ozone and ovalbumin, are summarized in Table 1.

**Intraepithelial mucosubstances.** The NTE of maxilloturbinates is normally void of secretory cells and saline-instilled, air-exposed animals have little or no stored mucosubstances in these tissues (Fig. 5A). By contrast, the RE of the septum normally consists of numerous goblet cells, and thus has high levels of stored mucosubstances (Fig. 5B). Repeated exposures to ovalbumin alone caused a significant increase in stored intraepithelial mucosubstances in the RE lining the nasal septum, but had no effect on stored mucosubstances in the NTE of maxilloturbinates. Ozone exposure alone caused no significant effect on the amount of intraepithelial mucosubstances in either the RE or the NTE. The combination of ozone and allergen however, caused the appearance of mucus-containing cells and a significant increase in the amount of intercellular mucosubstances in the NTE of maxilloturbinates (i.e., mucous cell metaplasia). Furthermore, ozone and allergen coexposure induced greater increases in intraepithelial mucosubstances in the RE lining the septum than those caused by ovalbumin alone.

**Epithelial DNA synthesis.** We have previously demonstrated that increased DNA synthesis in rat NTE is a reliable marker of injury/repair processes after exposure to ozone. In the present study, exposure to 0.5 ppm ozone for 1 or 3 days caused significant DNA synthesis in the NTE lining the maxilloturbinates, as indicated by an increase in epithelial cell staining positive for nuclear BrdU (Fig. 6A). Ozone exposure had no effect on DNA synthesis in the RE lining the septum (Fig. 6B). A single challenge with ovalbumin induced DNA synthesis in both the NTE and RE, but 3 days of challenges with ovalbumin were without effect in either tissue. The combination of ovalbumin and ozone induced significant BrdU labeling after 1 day in both the NTE and RE. After 3 days of ozone/ovalbumin coexposure, BrdU labeling was significant only in the NTE.

**Epithelial cell density.** Intranasal challenge with ovalbumin for 1 or 3 days had no effect on epithelial cell density in

### Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Inflammation</th>
<th>N/D</th>
<th>Cilia loss</th>
<th>Hypertrophy</th>
<th>Hyperplasia</th>
<th>SM</th>
<th>MCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ozone</td>
<td>NTE</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>NTE</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ozone and ovalbumin</td>
<td>NTE</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Note.* Relative histological responses: +, mild; ++, moderate; ++++, marked. N/D, necrosis/regeneration; SM, squamous metaplasia; MCM, mucous cell metaplasia.
of both the maxilloturbinate and the nasal septum (Fig. 8). Single or repeated exposures to ozone caused eosinophilic inflammation in the maxilloturbinites but not in the septum. In animals exposed once to ozone and challenged with ovalbumin, infiltration of eosinophils into the mucosa of maxilloturbinites was 3-fold greater than the significant accumulation caused by a single ovalbumin challenge alone. By comparison, eosinophilic responses in the septum after ozone exposure and ovalbumin challenge were similar to those observed after ovalbumin alone.

Neutrophils. A single challenge with ovalbumin induced a significant accumulation of neutrophils in the NTE and the underlying lamina propria of maxilloturbinites, and in the mucosa of the septum (Fig. 9). After three intranasal challenges

![Image](image_url)

**FIG. 6.** Effects of ozone and allergen exposures on cell labeling index in the NTE of maxilloturbinites (A) and RE of the septum (B). Animals were exposed to either air or 0.5 ppm ozone for 8 h and then challenged intranasally with either saline or ovalbumin. Twenty-four h after either a single exposure or after 3 daily exposures, animals were sacrificed and tissues collected and processed as described in Materials and Methods. Animals were injected with BrdU 2 h before sacrifice to label newly synthesized DNA. Data is expressed as mean ± SEM; n = 6; a, significantly different from respective control challenged with saline; b, significantly different from respective control exposed to air.

![Image](image_url)

**FIG. 7.** Effects of ozone and allergen exposure on epithelial cell density in the NTE of maxilloturbinites (A) and RE of the septum (B). Animals were exposed to either air or 0.5 ppm ozone for 8 h and then challenged intranasally with either saline or ovalbumin. Twenty-four h after either a single exposure or after 3 consecutive daily exposures, animals were sacrificed and tissues collected and processed as described in Materials and Methods. Data is expressed as mean ± SEM; n = 6; a, significantly different from respective control challenged with saline; b, significantly different from respective control exposed to air.

neither the NTE lining maxilloturbinites or the RE lining the septum (Fig. 7). Similarly, exposure to ozone for 1 day did not alter epithelial cell density in either tissue. However, 3 days of ozone exposure caused a significant increase in the density of epithelial cells in the NTE (epithelial hyperplasia). In rats given both ozone and ovalbumin, increased epithelial cell density was seen only in the NTE and only after 3 days (epithelial hyperplasia and squamous metaplasia). This increase in cell number was significantly greater than the epithelial hyperplasia in NTE caused by ozone alone after 3 days.

**Inflammatory cell Infiltration**

Eosinophils. Challenge with ovalbumin for either 1 or 3 days induced similar eosinophilic accumulations in the mucosa...
with ovalbumin, neutrophilic inflammation remained elevated in the septum, but was slightly less severe in the maxilloturbinates when compared to a single intranasal challenge. Exposure to ozone alone did not cause neutrophilic inflammation in either the maxilloturbinate or the septum at 24 h postexposure. Neutrophilic inflammation in response to ozone/ovalbumin was similar to that caused by ovalbumin alone.

**DISCUSSION**

Data from both human and animal studies suggest that ozone exacerbates pulmonary dysfunction and inflammation in asthmatics. In the present study, we used a morphometric approach to evaluate both nasal inflammatory and epithelial cell responses to ozone exposure in an animal model of allergic rhinitis. Our results demonstrate that (1) ozone enhances allergen-induced production and storage of mucosubstances in pre-existing secretory cells in nasal RE, (2) coexposure to allergen and ozone elicits MCM in the NTE, which is normally devoid of secretory cells, and (3) ozone and allergen exposure enhances eosinophilic inflammation in maxilloturbinates.

Exposure to ozone or allergen elicits distinct yet overlapping responses in the nose. For example, both agents cause a marked infiltration of inflammatory cells, epithelial cell injury, mucus secretion, and increased storage of epithelial mucosubstances (Harkema et al., 1997; Hotchkiss et al., 1997; Miadonna et al., 1999; Varney et al., 1992; Watanabe and Kiuna, 1998). Agent-specific differences exist in the nature of nasal alterations and inflammatory responses. In general, nasal responses to aller-
gens involve the RE, which consists of ciliated cells and preexisting secretory cells and is marked by recruitment and activation of eosinophils. Conversely, ozone elicits neutrophilic inflammation and mucous cell metaplasia in the epithelium of the lateral meatus, lined by NTE in the proximal nasal airways.

In the present study, ovalbumin challenge caused increases in DNA synthesis and stored mucosubstances in the RE lining the septum but not in the NTE of the maxilloturbinates. The increase in stored mucus in the RE was not due to increases in cell density. This is consistent with observations in nasal RE of humans with allergic rhinitis, where goblet cell density is unaltered after acute allergen challenge (Berger et al., 1997; Karlsson and Pipkorn, 1989). Our data suggest that preexisting secretory cells in the septum produce and store more mucosubstances in response to allergen instillation.

We also found that the combination of ozone exposure and ovalbumin challenge resulted in increased stored mucosubstances in the septum that were significantly greater than those induced by ovalbumin challenge alone. Again, cell density was not altered by these treatments, and therefore preexisting secretory cells contain increased volumes of stored mucus. These results demonstrate that ozone can potentiate the ovalbumin-induced alterations in the existing mucous apparatus in regions of the nose (i.e., in the RE) where ozone alone normally has no effect.

We have recently described in detail how repeated daily exposures to ozone cause inflammation, hyperplasia, and mucous cell metaplasia in the NTE of maxilloturbinates in Fisher F344 rats (Cho et al., 1999, 2000). Specifically, increased cell density is evident after one day of exposure and mucous cells appear by 2–4 days after repeated daily ozone exposures. We have also reported that 4 days of ozone exposure of Brown Norway rats result in hyperplasia but not in MCM in the NTE (Hotchkiss et al., 1999). We extended these findings in the present study by evaluating inflammatory and epithelial changes after 1 day of ozone exposure, at a time when ozone-initiated, premetaplastic events occur (i.e., inflammation, epithelial cell density). In the present study we found that intranasal challenge with allergen enhanced ozone-induced hyperplastic responses (Fig. 7), and that the combination of allergen and ozone caused MCM (Fig. 5) in the NTE of Brown Norway rats. These results suggest that the allergen acts either to increase ozone-initiated pathways or to initiate distinct pathways that act synergistically with ozone. Similar hypotheses can be proposed for the effect of ozone to augment allergen responses in the RE of the septum. The results of the present study suggest that each agent enhances the site-specific epithelial responses of the other.

As a first step in elucidating the mechanism(s) for these responses, we evaluated inflammatory cell influx at times that precede (i.e., after a single instillation or exposure) and that are concurrent (i.e., after 3 such treatments) with epithelial cell changes. Ozone exposure alone had no effect on neutrophil recruitment into the septum, and in fact, partially blocked the accumulation of neutrophils elicited by allergen challenge (Fig. 9B). Thus, the ability of ozone to enhance production of mucus in preexisting secretory cells is not due to a numeric increase in infiltrated neutrophils.

Associations exist between the presence of eosinophils in airway mucosa and allergen-induced epithelial cell responses and hypersensitivity (Blyth et al., 1996; Durham et al., 1992; Elwood et al., 1992). We report here a relationship between eosinophil accumulation and increased mucosubstances in the RE of the septum. Although ozone exposure enhances the increase in intraepithelial mucosubstances in the septum, it has no effect on the eosinophil accumulation in these tissues. Taken together, if the presence of either eosinophils or neutrophils underlies the mechanism of ozone-induced enhancement of mucous cell responses in the RE, our data suggest their presence may be qualitative rather than quantitative. That is, ozone or a mediator of ozone might activate these inflammatory cells or alter their function such that they contribute to mucous cell responses.

Repeated coexposures to ozone and ovalbumin induced ep- thelial hyperplasia and mucous cell metaplasia in the NTE of maxilloturbinates where there are normally no mucus-contain- ing cells (Figs. 5 and 7). The only inflammatory cell response after coexposure that differed from a single agent exposure was a synergistic increase in eosinophils after a single coexposure (Fig. 8A). One exposure to ozone alone also caused increases in eosinophilic inflammation, but did not lead to significant increases in stored mucosubstances. Repeated ozone exposures did result in hyperplasia, albeit less than that produced by coexposure to ozone and allergen. It is possible that the magni- tude of eosinophilic inflammation early after treatments dic- tates the rate and degree of epithelial cell changes in NTE (i.e., hyperplasia and metaplasia). In the present study, the rate of DNA synthesis after a single coexposure and the cell density achieved after multiple coexposures both correlate with the early eosinophilic response. Thus, the stronger eosinophilic response induced by ozone and allergen may portend a more robust hyperplastic and metaplastic response in the NTE.

Our work in Fisher F344 rats suggests that either neutrophil-derived products or cell–cell interactions of neutrophils and epithelial cells mediate, in part, the ozone-induced MCM (Cho et al., 1999). In this regard, neutrophil products such as platelet activating factor (PAF), elastase, and reactive oxygen species have been implicated by others in the activation of mucin protein coding genes in airway epithelial cells (Lou et al., 1998; Voynow et al., 1999). MCM or the induction of mucin genes in airway cells can be mediated by factors such as interleukin-4 (IL-4), IL-9, IL-13, prostaglandin E₂, tumor necrosis factor, 15-HETE, and members of the epidermal growth factor family (Borchers et al., 1999; Dabbagh et al., 1999; Takeyama et al., 1999). It is notable that all of these factors can be secreted or produced by eosinophils. As such, the eosinophil
may secrete mediators that are responsible in part for MCM in nasal epithelium.

Recent reports, however, suggest that eosinophils are not required for allergen-induced MCM in the lower airways of mice (Cohn et al., 1999; Haile et al., 1999). Likewise, it is possible that eosinophils in the nasal airways of exposed rats in the present study are not necessary for the concurrent epithelial cell changes. Mast cells and T lymphocytes also produce mediators which promote MCM in airways (e.g., IL-4, IL-9) during allergic inflammation. Furthermore, ozone alone is capable of activating mast cells and T lymphocytes to release putative mediators of metaplasia (Chen et al., 1995; Schierhorn et al., 1999). Thus, redundant signals for epithelial cell differentiation are likely present after ozone and allergen exposures. Future studies in which eosinophils, neutrophils, or mast cells are depleted or their activation inhibited before ozone and allergen challenge are needed to understand the contribution of these inflammatory cells to the epithelial changes we have described here.

Previous descriptions of upper airway inflammation after ozone and allergen challenge have been limited to the analysis of nasal lavage. The present study provided a morphologic approach to understanding the temporal involvement of inflammatory cells with epithelial changes induced by coexposure of an oxidant pollutant and an inhaled allergen. Specifically, we have documented increased mucus storage, hyperplasia, MCM, and the relationship of these epithelial changes with eosinophilic and neutrophilic inflammation. Based on the changes in the nasal mucous apparatus in different nasal airway epithelium populations, our results suggest that each stimulus (i.e., ozone and allergen) enhances the effects of the other. As such, people with allergic rhinitis may be more sensitive to the health risks of ozone exposure than are normal, noncompromised individuals. Further studies are required to identify the cellular and molecular mechanisms responsible for the enhanced mucous cell responses after ozone and allergen coexposure.

**ACKNOWLEDGMENT**

Research for this paper was supported in part by NIH grant HL59391.

**REFERENCES**


and 0.8 ppm ozone on rat nasal and nasopharyngeal epithelial mucous


