Endotoxin Enhancement of Ozone-Induced Mucous Cell Metaplasia Is Neutrophil-Dependent in Rat Nasal Epithelium

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Ozone, the primary oxidant gas in photochemical smog, causes neutrophilic inflammation and mucous cell metaplasia (MCM) in the nasal transitional epithelium (NTE) of rats and monkeys. Bacterial endotoxin is another common airborne agent that induces acute neutrophilic inflammation, but not MCM, in NTE. It does, however, enhance ozone-induced MCM in rat nasal airways (Fanucchi et al., 1998, Toxicol. Appl. Pharmacol. 152, 1–9). In the present study, F344 rats exposed to filtered air or 0.5 ppm ozone (8 h/day for 3 days) were intranasally instilled with sterile saline or 100 μg endotoxin 24 h and 48 h after the third ozone exposure. To determine the role of neutrophilic inflammation in endotoxin-induced potentiation of the MCM caused by ozone, half of the rats were depleted of circulating neutrophils prior to saline or endotoxin instillations. Rats were killed 6 h or 3 days after the last intranasal instillation, and nasal tissues were processed for (1) light microscopy and morphometric analysis to determine the number of infiltrating neutrophils and the volume amount (density) of stored mucosubstances in the NTE, and (2) quantitative RT-PCR analysis of steady-state mucin gene (rMuc-5AC) mRNA levels in the NTE. Endotoxin induced a transient influx of neutrophils in both air- and ozone-exposed rats that was completely blocked by neutrophil depletion. Endotoxin increased rMuc-5AC mRNA levels in the NTE of ozone-exposed rats. Neutrophil depletion, however, had no effect on endotoxin-induced upregulation of mucin gene mRNA levels. Endotoxin enhanced the ozone-induced increase in stored mucosubstances (4-fold increase), but only in neutrophil-sufficient rats. These data indicate that endotoxin enhancement of ozone-induced upregulation of rMuc-5AC mRNA levels is neutrophil-independent, while its effects on intraepithelial production and storage of mucus glycoproteins is dependent on the presence of neutrophils.

Key Words: ozone; endotoxin; mucous cell metaplasia; neutrophils; nasal epithelium.

Inhalation of ozone, the primary oxidant pollutant in photochemical smog, elicits a constellation of epithelial-cell responses in the upper airways of humans. Among these effects are genotoxicity, inflammation, hypersecretion and production of mucus, epithelial hyperplasia, and metaplasia (Calderon-Garciduenas et al., 1995, 1999; Frischer et al., 1993; Graham et al., 1988). In previous studies we have characterized similar inflammatory and epithelial responses to ozone exposure in the nasal airways of the laboratory rat (Harkema and Hotchkiss, 1994; Harkema et al., 1997). In particular, we have focused on the cellular and molecular mechanisms involved in mucous cell metaplasia (MCM)—the appearance of mucus goblet cells in areas that are normally devoid of these secretory cells, such as the nasal transitional epithelium (NTE). The sequence of events leading to ozone-induced MCM includes neutrophilic inflammation, epithelial necrosis, and exfoliation, followed by epithelial-cell proliferation and hyperplasia, and finally the appearance of mucous cells (i.e., MCM). We have also demonstrated that the inflammatory component (i.e., neutrophils) in this process is necessary for full metaplastic responses. In this regard, blockade of neutrophil recruitment into the NTE by treatment with either corticosteroids or neutrophil-depleting antibodies can markedly attenuate MCM after acute ozone exposure (Cho et al., 2000; Hotchkiss et al., 1998b). However, the underlying cellular mechanisms of ozone-induced MCM remain unknown.

Endotoxins are lipopolysaccharide-protein components of the cell wall of Gram-negative bacteria. Bacterial endotoxins are potent inflammagens, and are commonly found on airborne vectors such as organic dusts generated from agricultural or industrial sources (Rylander, 1995; Simpson et al., 1999). Inhalation of endotoxin can elicit inflammatory cell recruitment and epithelial cell injury in humans and animals (Sandstrom et al., 1992; van Helden et al., 1997). We have previously described endotoxin-induced epithelial and inflammatory responses in the rodent nasal airways (Harkema and Hotchkiss, 1991, 1993). Intranasal instillation of endotoxin causes acute neutrophilic rhinitis that is accompanied by hypersecretion of mucus from secretory cells of the respiratory epithelium (RE), but engenders only acute inflammation (i.e., neutrophil influx) without MCM in the NTE. More recently, we have demonstrated that the presence of airway endotoxin enhances the development of ozone-induced MCM in the NTE (Cho et al., 1999a; Fanucchi et al., 1998). Endotoxin promotes ozone-induced metaplastic responses in the NTE where alone it causes only acute inflammation but not metaplasia. The cellular mechanisms of endotoxin’s ability to augment ozone-induced epithelial cell responses in this tissue are unknown. We
have shown in vitro that endotoxin can induce mucin gene expression in cultured nasal explants (Hotchkiss et al., 1998a). In addition, several soluble mediators that are produced by endotoxin-activated inflammatory cells (e.g., tumor necrosis factor, interleukin-1, platelet-activating factor, and proteases) have been demonstrated to promote epithelial cell hyperplasia, MCM, and the expression of genes that code for mucin proteins (Borchers et al., 1999; Lou et al., 1998; Voynow et al., 1999). Thus endotoxin might promote MCM by both direct and indirect pathways.

Because ozone-induced MCM is partially dependent on neutrophils, we hypothesized that enhancement by endotoxin of ozone-induced MCM is also dependent on the influx of neutrophils. To test this hypothesis, we used an antibody directed against rat polymorphonuclear leukocytes (PMNs) to deplete animals of circulating neutrophils and thereby block neutrophilic inflammation, caused by endotoxin, in nasal airways. In the present study we demonstrated, using histologic, morphometric, and immunohistochemical approaches, that the enhancement of ozone-induced MCM by endotoxin is neutrophil-dependent. Furthermore, we describe the nature of this dependence in relation to concurrent hyperplasia and mucin-specific gene expression that precedes the ozone-induced MCM in the NTE.

MATERIALS AND METHODS

Animals. Ninety-six male F344/N rats (Harlan Sprague-Dawley, Indianapolis, IN), 10–12 weeks of age, were randomly assigned to one of 16 experimental groups (n = 6/group). Rats were free of pathogens and respiratory disease, and used in accordance with guidelines set forth by the All-University Committee on Animal Use and Care at Michigan State University. Animals were housed two per cage in polycarbonate boxes, on Cell-Sorb Plus bedding (A&W Products, Cincinnati, OH) covered with filter lids, and had free access to tap water and food (Tek Lad 1640, Harlan Sprague Dawley, Indianapolis, IN). Room lights were set on a 12-h light/dark cycle beginning at 6:00 a.m., and temperature and relative humidity were maintained between 21–24°C and 40–55%, respectively. During the inhalation portion of the study, rats were housed individually in rack-mounted stainless steel wire cages in the middle of the cage racks. The concentration of ozone during exposures was 0.5 ppm, ozone was generated with an OREC model O3V1-O ozone generator (Ozone Research and Equipment Corp., Phoenix, AZ) using compressed air as a source of oxygen. Total airflow through the exposure chambers was monitored throughout the exposure using 2 Dasibi 1003 AH classifiers (Ozone Research and Equipment Corp., St. Louis, MO) using a post-mounted homogenizer with a 5-mm nozzle. Total airflow through the exposure chambers was maintained at 0.5 m3/min (15 chamber air change/h). The concentration of ozone within the breathing zone of rats within the middle chambers was monitored throughout the exposure using 2 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 of the cage racks. The concentration of ozone during exposures was 0.5 ± 0.011 ppm (mean ± SEM) for ozone chambers and less than 0.02 ppm for control chambers.

Neutrophil depletion (Day 3). Immediately after the last inhalation exposure, rats were removed from the chambers, anesthetized with 4% halothane in oxygen, and injected intraperitoneally with either 1 ml of rabbit anti-rat PMN antiserum (Accurate Scientific Corp., Westbury, NJ), or normal rabbit serum (control serum). Treatment with the antiserum is known to deplete circulating neutrophils below 1% of normal levels by 12 h and depletion persists for up to 5 days (Snipes et al., 1995). After treatment with either control serum or antiserum, animals were placed in polycarbonate boxes with filter lids for the duration of the experiment.

Endotoxin instillations (Days 4 and 5). Fourteen h after treatment with PMN antiserum (Day 4), animals were intranasally instilled with endotoxin by methods described in Harkema and Hotchkiss, 1991. Briefly, rats were anesthetized with 4% halothane in oxygen, and 50 μl of endotoxin (1 mg/ml, lipopolysaccharide from *Pseudomonas aeruginosa* Serotype 10; Sigma Chemical Co., St. Louis, MO) in pyrogen-free saline was instilled into each nasal passage of 48 rats (total dose of 100 μg). The other 48 rats were instilled with pyrogen-free saline. Instillation procedures were repeated 24 h later on Day 5.

Necropsy and tissue preparation (Days 5 and 8). Forty-eight rats were killed 6 h after the second intranasal instillation of endotoxin. The remaining forty-eight rats in the study were killed 3 days after the last instillation. Rats were anesthetized with sodium pentobarbital (50 mg/kg), a midline laparotomy was performed, and 3 ml of blood was drawn from the abdominal vena cava into a Vacutainer containing EDTA as an anticoagulant. Total white blood cells in whole blood were enumerated with a Serono-Baker System 9000 automated cell counter (Serono-Baker Diagnostics, Allentown, PA). Total blood neutrophils were determined by their percent occurrence in at least 100 white blood cells counted in smears stained with Diff-Quik (Baxter, McGaw Park, IL). Rats were killed by exsanguination via the abdominal aorta. Immediately after death, the head of each rat was removed from the carcass and the lower jaw and skin were removed. The nasal airways were exposed by splitting the skull in a sagittal plane adjacent to the midline. One-half of the head was immersed in a large volume of zinc-formalin (AnaTech, Kalamazoo, MI) for at least 48 h. After fixation, this half of the head was decalcified in 13% formic acid for 4 days, and then rinsed in distilled water for 4 h. A tissue block was removed from the anterior nasal cavity by making 2 cuts perpendicular to the lower jaw and skin were removed. The nasal airways were exposed by splitting the skull in a sagittal plane adjacent to the midline. One-half of the head was immersed in a large volume of zinc-formalin (AnaTech, Kalamazoo, MI) for at least 48 h. After fixation, this half of the head was decalcified in 13% formic acid for 4 days, and then rinsed in distilled water for 4 h. A tissue block was removed from the anterior nasal cavity by making 2 cuts perpendicular to the hard palate, the first immediately posterior to the upper incisors, and the second at the level of the incisive papilla (Fig. 2A). The tissue blocks were embedded in paraffin, and 5–6 μm thick sections were cut from the anterior surface. Nasal sections were stained with hematoxylin and eosin (H&E) for routine histology or with Alcian Blue (pH 2.5)/Periodic Acid-Schiff (AB/PAS) to detect intraepithelial mucous substances.

The maxilloturbinate from the other half of the head was removed by microdissection and homogenized in 0.5 ml Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) using a post-mounted homogenizer with a 5-mm generator (Model 250, Pro-Scientific, Inc., Monroe, CT). Samples were kept at −80°C until further processing for RNA isolation.

Morphometry of stored intraepithelial mucous substances. To estimate the amount of the intraepithelial mucous substances in NTE lining maxilloturbinates, the volume density (Vs) of AB/PAS-stained mucous substances was quantified using computerized image-analysis and standard morphometric techniques.
and is expressed as nanoliters (nl) of intraepithelial mucosubstances per mm\(^2\) of basal lamina.

**Morphometry of epithelial cell numeric density.** 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 endotoxin on neutrophil influx within the NTE of maxilloturbinate was determined in H&E-stained sections by counting the total number of neutrophils within the nasal mucosa (area between the turbinate bone and airway lumen) and dividing by the total length of the basal lamina. Neutrophils were identified by morphologic characteristics that included their size, darkly stained multilobed nuclei, and clear cytoplasm with dust-like granules.

**RNA isolation.** Total RNA was isolated from microdissected, homogenized maxilloturbinate by following the method of Chomczynski and coworkers (Chomczynski and Mackey, 1995; Chomczynski and Sacchi, 1987). Isolated RNA pellets were resuspended in nuclease-free water and incubated with DNase solution (100 units rRNasin [Promega, Madison, WI], 100 mM DTT [Life Sciences Technology Inc., Grand Island, NY], and 10 units DNase I [Boehringer Mannheim, Indianapolis, IN] in 5x transcription buffer [Promega]) for 45 min at 37°C. The RNA was extracted sequentially with equal volumes of phenol/chloroform/isomyl alcohol (25:24:1) and chloroform/isomyl alcohol (24:1), and precipitated with 10 M ammonium acetate and isopropanol. The pellet was washed with 75% ethanol, air dried, and resuspended in nuclease-free water containing rRNasin (40 units/100 µl). RNA concentrations were determined with a fluorescent RNA-binding assay (RiboGreen; Molecular Probes, Eugene, OR), using a SpectraMax GEMINI spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA).

**Quantitative RT-PCR.** Steady state levels of Muc-5AC mRNA were determined in rat maxilloturbinate from ozone-exposed rats using a quantitative RT-PCR technique. Muc5AC is a specific protein for secretory epithelial cells and not in other cells of the maxilloturbinate. Therefore, RT-PCR of maxilloturbinate RNA was used to estimate the Muc-5AC mRNA that is present in epithelium. The quantitative RT-PCR technique employs a recombinant competitor RNA (rcRNA), used as an internal standard (IS), which is reverse-transcribed and amplified in the same tubes as the target sequence (rMuc-5AC). The IS rcRNA was synthesized as described previously (Fanucchi et al., 1999). The IS contains the same sequences recognized by the amplification primers for rMuc-5AC, but has a different-sized intervening sequence and therefore yields a different-sized RT-PCR product. The concentration of Muc-5AC mRNA was estimated by adding increasing, known amounts of IS (in numbers of molecules) to the RT-PCR mixtures that contain a constant, unknown amount of sample RNA. Because both the IS and sample RNA are amplified at the same rate, this procedure results in an absolute experimental readout (molecules of target gene mRNA per unit sample).

RT-PCR for rMuc-5AC was performed as outlined by Gilliland and coworkers (1990a,b), except that known amounts of the IS rcRNA were reverse-transcribed into complementary DNA (cDNA) in a volume of 20 µl containing PCR buffer plus 5 mM MgCl\(_2\), 1 mM each dNTP, 10 units rRNasin, 125 ng oligo(dT)\(_{12-18}\) (Becton Dickinson, Bedford, MD), 100 ng total RNA from maxilloturbinate, and 40 units of MMLV reverse transcriptase (Promega). For each RNA sample from individual animals, a known concentration of IS rcRNA molecules was added that was similar in concentration to the RNA samples. This was determined in a preliminary range-finding experiment using pooled samples of each experimental group, to be between 10\(^{6}\)–10\(^{7}\) molecules per sample. A standard curve was also prepared by adding 10-fold serial dilutions of the IS (10\(^{9}\)–10\(^{10}\) molecules per tube) to a constant amount of RNA (pooled from all samples). All RNA samples were then incubated at 42°C for 15 min, followed by an incubation at 95°C for 4 min. A PCR master-mix consisting of PCR buffer, 4 mM MgCl\(_2\), 6 pmol each of rMuc-5AC forward (5’-CATCATTCTCTGTAGCAGTGAGG-3’) and reverse (5’-GTTACCCAGGTCTCACCTACTCCG-3’) primers, and 1.25 units Taq DNA polymerase were added to the cDNA samples, for a final volume of 50 µl (Taq

\[ V_s = \frac{\text{area of AB/PAS stained material}}{4/\pi \times \text{length of basal lamina}} \times \text{section thickness} \times 1000 \]
polymerase was added to the PCR master-mix after it had been heated to 85°C for 5 min. Samples were then immediately heated to 95°C for 4 min and then cycled 36 times at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, after which an additional final extension step at 72°C for 10 min was included.

PCR products (10 µl) were electrophoresed on a 3% agarose gel (NuSieve 3:1; FMC Bioproducts, Rockland, ME) and visualized by ethidium-bromide staining. Densitometry was carried out using a Bio-Rad ChemiDoc image acquisition system and Quantity One (Ver. 4.0) quantitation software (Bio-Rad, Hercules, CA), running on a Dell OptiPlex GX1 computer. The density ratio of the rMuc-5AC PCR product band to the corresponding IS PCR product band present in each sample was determined as described by Gilliland and colleagues (1990a,b). A standard curve was constructed by plotting the log of the density ratio (i.e., rMuc-5AC PCR product band/IS PCR product band) versus the log of IS serial dilution concentrations added to the standards (i.e., $10^{0}$–$10^{9}$ molecules/tube). Linear regression was performed on the standard curve to determine slope and y-intercept, which yielded the amount of rMuc-5AC mRNA (molecules) present in the pooled RNA standard sample when mRNA/IS = 1.

The rMuc-5AC mRNA value was then divided by each IS serial dilution concentration to arrive at an “actual” ratio. A transformed standard curve was then calculated by plotting the original density ratio versus the actual ratio, and linear regression was performed. The transformed standard curve was calculated to linearize the single point measurements of the experimental samples, which were obtained with the following equation:

$$\text{actual mRNA (molecules) = actual IS} \times 10^{\text{log(density ratio) - y-intercept/slope}}$$

Data are expressed as the number of rMuc-5AC mRNA molecules per ng of total sample RNA that was added to the RT-PCR reaction.

Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). Data were 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 $p \leq 0.05$.

RESULTS

Effect of Rat PMN Antiserum on Blood and Tissue-Associated Neutrophils

Treatment of rats with PMN antiserum decreased circulating neutrophils to less than 1% of circulating white blood cells in rats killed 6 h after endotoxin instillations, irrespective of endotoxin instillation or ozone exposure (Table 1). Circulating neutrophils remained depressed for the duration of the experiment (3 days after endotoxin instillations).

Rats treated with control serum had few, if any, neutrophils within the NTE 6 h after the last instillation of saline, regardless of ozone exposure. Intranasal instillation of endotoxin, however, induced a dramatic increase in epithelial and subepithelial neutrophils after 6 h (34 neutrophils/mm basal lamina) that returned to control levels by 3 days. The combination of ozone exposure and endotoxin instillation caused an increase in neutrophils in the NTE that was similar to that caused by endotoxin alone (38 neutrophils/mm basal lamina). By 3 days, neutrophilic inflammation in rats exposed to ozone and instilled with endotoxin was similar to unexposed rats instilled with saline. The NTE of neutrophil-depleted rats were devoid of neutrophils in all experimental groups at 6 h and at 3 days (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>PMN antiserum</th>
<th>Circulating neutrophils (Cells/100 µl blood)</th>
<th>Tissue neutrophils (Cells/mm basal lamina)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h</td>
<td>3 days</td>
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<tr>
<td></td>
<td>6 h</td>
<td>3 days</td>
</tr>
<tr>
<td>Control serum</td>
<td>760 ± 271‡</td>
<td>881 ± 103‡</td>
</tr>
<tr>
<td>Saline/ozone</td>
<td>1005 ± 302‡</td>
<td>0 ± 0‡</td>
</tr>
<tr>
<td>Endotoxin/ozone</td>
<td>1034 ± 387‡</td>
<td>34.5 ± 4.5‡†</td>
</tr>
<tr>
<td>Endotoxin/air</td>
<td>906 ± 223‡</td>
<td>37.7 ± 6.2‡*</td>
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<tr>
<td><strong>PMN antiserum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline/ozone</td>
<td>5 ± 4**‡</td>
<td>10 ± 9**‡</td>
</tr>
<tr>
<td>Saline/ozone</td>
<td>0.5 ± 0.5**‡</td>
<td>0.23 ± 0.56‡</td>
</tr>
<tr>
<td>Endotoxin/air</td>
<td>4 ± 5**‡</td>
<td>0 ± 0‡</td>
</tr>
<tr>
<td>Endotoxin/ozone</td>
<td>7 ± 8**‡</td>
<td>0 ± 0‡</td>
</tr>
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Note. Animals were treated with control serum or rat PMN antiserum after ozone exposures but prior to endotoxin instillations.

* Significantly different from respective group given instillations of saline.
** Significantly different from respective group treated with control serum.

Nasal Histopathology

**Filtered air-exposed rats.** Both neutrophil-sufficient and neutrophil-depleted rats that were exposed to filtered air (filtered air-controls), and had also received intranasal instillations of saline, exhibited no exposure-related microscopic alterations in the examined nasal tissues at either 6 h or 3 days after the last instillation. In contrast, filtered air-exposed, neutrophil-sufficient rats that were instilled with endotoxin and killed 6 h post-instillation had a markedly diffuse neutrophilic rhinitis characterized by a conspicuous influx of neutrophils in the lamina propria and the overlying surface epithelium of the nasal mucosa. An associated neutrophilic exudate was often conspicuously present in the adjacent nasal airway lumens. The inflammatory cell infiltrate was most severe in the mucosal tissues that lined the lateral and middle meatus and contained NTE or respiratory epithelium. Only a mild inflammatory cell response was evident in the olfactory mucosa lining the dorsal meatus. In addition, there was no histologic evidence of degeneration or necrosis of the NTE, respiratory epithelium, or olfactory epithelium in these animals.

An endotoxin-induced neutrophilic rhinitis was predictably absent in the filtered air-exposed rats that were neutrophil-depleted and killed at 6-h post-instillation. In addition, there was no morphologic alteration in the NTE. There was a mild to moderate mucous cell hypertrophy, due to an increase in intracellularly stored mucosubstances in the respiratory epithelium lining the proximal aspect of the mid-nasal septum.

Filtered air-exposed and neutrophil-sufficient or -deficient rats that received endotoxin instillations and were killed 3 days after the end of the instillations had only a few mononuclear cells (lymphocytes and monocytes) in the lamina propria beneath the NTE and respiratory epithelium lining the lateral and
middle meatus of the proximal nasal cavity. The only treatment-induced change in the NTE was a minimal to mild hyperplasia in neutrophil-sufficient rats exposed to endotoxin. In addition, moderate hypertrophy of mucous cells with increased amounts of mucosubstances was present in the respiratory epithelium lining the mid-nasal septum of both neutrophil-sufficient and -deficient rats exposed to endotoxin. No other microscopic alterations were present in either the respiratory or transitional epithelium.

**Ozone-exposed rats.** The principal nasal lesion in the ozone-exposed and neutrophil-sufficient rats that received intranasal saline instillations and were sacrificed 6-h post-instillation was a marked epithelial hyperplasia of the NTE lining the lateral meatus and covering the mucosal surface of the proximal maxilloturbinates, lateral wall, and lateral aspects of the nasal turbinates. In addition, these rats had a minimal to mild influx of mononuclear cells with lesser numbers of eosinophils and only an occasional neutrophil in the lamina propria of the transitional mucosa lining the lateral meatus. Mild to moderate hypertrophy of mucous cells in the respiratory epithelium lining the mid-septum was also present in these rats.

Neutrophil-sufficient rats that were similarly exposed to ozone and intranasally instilled with saline, but were sacrificed 3 days after the last saline instillation, also had a markedly hyperplastic NTE. There was also a minimal to mild mucous cell metaplasia in the NTE of these ozone-exposed rats (Fig. 3). Only a minimal to mild increase in mononuclear cells and

**FIG. 3.** Light micrographs of maxilloturbinates from rats exposed to filtered air and killed 3 days after intranasal instillation with saline (A, control group), or exposed to filtered air and killed 3 days after intranasal instillation with endotoxin (B, endotoxin alone), or exposed to 0.5 ppm ozone and killed 3 days after intranasal instillation with saline (C, ozone alone). Tissues were stained with AB/PAS to detect acidic and neutral mucosubstances. Arrows indicate AB/PAS-stained intraepithelial mucosubstances in mucous cells; e, epithelium (NTE); b, bone; v, blood vessel.

**FIG. 4.** Light micrographs of H&E-stained maxilloturbinates from rats exposed to 0.5 ppm ozone, treated with either control serum (A) or with PMN antiserum (B), and killed 3 days after intranasal instillations with endotoxin. Arrows indicate infiltrated neutrophils in the lamina propria and epithelium; e, epithelium (NTE); tb, bone; v, blood vessel.
Acute neutrophilic rhinitis and conspicuous nasal epithelial hyperplasia were the principal nasal lesions in ozone-exposed, neutrophil-sufficient rats that had also received intranasal instillations of endotoxin and were sacrificed 6 h after the last instillation (Fig. 4). Like in all of the other ozone-exposed animals, their epithelial hyperplasia was restricted to the NTE lining the lateral meatus and covering the maxilloturbinate, lateral wall, and lateral aspects of the nasal turbinates in the proximal nasal passage. In contrast, the endotoxin-induced rhinitis was not site-specific, and was evident in all of the mucosal tissues in the proximal nasal cavity. However, like the other endotoxin-instilled and neutrophil-sufficient rats killed 6 h after the last instillation, the inflammatory response was most severe in the mucosa covered by NTE and respiratory epithelium. Though neutrophils were the predominant inflammatory cells, lymphocytes, monocytes, and lesser numbers of eosinophils were scattered in the lamina propria underlying the hyperplastic NTE.

Ozone-exposed, endotoxin-instilled, and neutrophil-deficient rats that were sacrificed at 6 h after the last instillation had the characteristic ozone-induced hyperplasia of the NTE lining the lateral meatus (Fig. 4). However, there was no neutrophilic rhinitis in these rats such as that present in the endotoxin-instilled rats that were neutrophil-sufficient and were sacrificed at a similar time post-instillation (Fig. 4). The only inflammatory lesion was a minimal to mild influx of lymphocytes, monocytes, and eosinophils in the lamina propria underlying the hyperplastic NTE.

The principal nasal lesions in ozone-exposed, neutrophil-sufficient rats that were instilled with endotoxin and sacrificed 3 days post-instillation were moderate to marked mucous cell metaplasia and epithelial hyperplasia in the NTE that lines the lateral meatus of the proximal nasal airway (Fig. 5). A mild mononuclear-cell influx with a few eosinophils and neutrophils in the lamina propria accompanied the regionally restricted epithelial proliferation and differentiation. Interestingly, neutrophil-deficient animals that were similarly exposed and instilled with endotoxin had similar nasal lesions, with the noticeable difference that there was no or minimal mucous cell metaplasia in the NTE (Fig. 5).

Mucous Cell Metaplasia

There were no significant increases in stored mucosubstances in NTE in any group at 6 h after the last intranasal instillation (Fig. 6A). A minor, yet statistically insignificant increase (4-fold) in stored mucosubstances in ozone-exposed, neutrophil-sufficient animals was evident and is consistent with the beginning of MCM at this time point. Three days after instillations, MCM was induced in saline instilled/ozone-exposed animals compared to those exposed to filtered air and instilled with saline (40-fold increase in stored mucosubstances; Fig. 6B). The combination of ozone exposure followed by endotoxin instillation, however, caused a dramatic, synergistic increase in intraepithelial mucosubstances with respect to either ozone (5-fold greater) or endotoxin (18-fold greater) alone. In neutrophil-depleted animals, the MCM caused by ozone exposure and endotoxin instillation was blocked completely, and intraepithelial amounts of mucosubstances were similar to those measured in saline-instilled, filtered air-exposed animals.

Epithelial Cell Density

Ozone alone caused a significant increase (40%) in NTE cell density (epithelial hyperplasia) in maxilloturbinates of neutrophil-sufficient animals 6 h after exposures (Fig. 7A). Epithelial hyperplasia in the NTE was significantly less in endotoxin-instilled, ozone-exposed animals killed at 6 h when compared to saline instilled, ozone exposed animals. In neutrophil-depleted rats, ozone exposure caused increased cell density irrespective of intranasal instillation.

Ozone-induced hyperplasia in neutrophil-sufficient animals was still evident 3 days after exposures, and endotoxin instillation alone caused hyperplasia compared to saline-instilled animals at this time point (Fig. 7B). Hyperplasia was induced in endotoxin-instilled, ozone-exposed animals and was greater than that caused by either stimulus given alone. In neutrophil-depleted rats at this time point, ozone exposure caused increased cell density irrespective of intranasal instillation. That is, endotoxin did not cause a further increase in cell density in ozone-exposed, neutrophil-depleted animals.

Mucin (rMuc-5AC) mRNA Expression

Six h after instillation, endotoxin caused a significant increase in Muc-5AC mRNA expression compared to ozone, which was unaffected by depletion of circulating neutrophils (Fig. 8A). By 3 days after instillations, differences in mRNA levels between experimental groups were not statistically significant (Fig. 8B).

DISCUSSION

Ozone exposure causes MCM in rat nasal epithelium that is preceded sequentially by neutrophilic inflammation, cell injury, hyperplasia, and mucin-specific gene expression. The presence of endotoxin in upper airways also promotes neutrophilic inflammation in NTE, but not mucous cell metaplasia. We have recently shown that endotoxin can dramatically augment ozone-induced metaplastic responses by unknown mechanisms (Fanucchi et al., 1998). The present study demonstrates that neutrophils are required for endotoxin to enhance mucous cell metaplasia initiated by ozone and that they do not modulate MCM in this model by inducing expression of genes responsible for the production of mucin apoprotein. Specifically, depletion of circulating neutrophils blocked MCM but had no effect on mucin-specific gene expression.

Early and transient inflammation, notably the presence of...
neutrophils in the NTE and the underlying submucosa, is required for metaplastic response to ozone exposure (Cho et al., 1999a). In the current study, endotoxin was given 24 h after 3 daily ozone exposures, at a time when the presence of ozone-induced inflammation in the NTE has subsided (Cho et al., 1999b). Our results show that endotoxin’s enhancement of MCM is accompanied by a significant influx of neutrophils that are not present with ozone exposure alone at this post-exposure time (i.e., 2 days after ozone exposures). Thus, the dosing protocol of ozone (3 days) followed by endotoxin (2 days) prolongs the presence of neutrophils in the NTE. By depleting animals of neutrophils after the ozone-induced inflammation had subsided, we were able to isolate the effects of endotoxin-induced neutrophilic influx on the ozone-induced MCM. Both the increase in mucosal neutrophils and MCM caused by endotoxin were blocked completely in neutrophil-depleted animals. This suggests that the neutrophilic influx caused by endotoxin instillation was responsible for enhancement of the MCM caused by ozone.

Airway endotoxin causes a more robust recruitment of neutrophils in upper airways than does exposure to ozone. Though not enumerated in the present study, we have observed previously that neutrophil accumulation in maxilloturbinates during acute ozone exposures are routinely 4–10-fold less than those elicited by endotoxin at the same time points (Cho et al., 1999b; Fanucchi et al., 1998; Hotchkiss et al., 1998b). In addition, the activation status of neutrophils after endotoxin or ozone treatments may be different. Endotoxin and ozone likely activate both similar and dissimilar inflammatory pathways that can affect neutrophil function. In this regard, the oxidant nature of ozone might induce oxidative stress in epithelial cells and lead to the activation of inflammatory cells, whereas endotoxin activates cells after receptor binding. We have shown that neutrophil recruitment into the NTE diminishes with repeated ozone exposures such that by the fourth or fifth day of repeated exposures, the numbers of infiltrated neutrophils are near control levels. It is notable that endotoxin can initiate a new round of neutrophilic infiltration at a time when ozone-induced signals for neutrophil recruitment have waned (i.e., at 4 and 5 days). This observation suggests that distinct chemotactic pathways (i.e., chemokines, cytokines) are invoked after treatment with endotoxin compared to repeated ozone exposure. Therefore, the activation status of endotoxin-elicited neutrophils, in addition to their cellular density, probably determines their contribution to MCM.

In addition to neutrophilic inflammation, ozone-induced MCM is always preceded by hyperplasia of epithelial cells in the NTE (Cho et al., 1999b; Harkema and Hotchkiss, 1994; Harkema et al., 1997). The hyperplastic response is maximal after 3 days of ozone, which is the same time, in the current study, when endotoxin and its accompanying inflammation were introduced. Endotoxin caused a decrease in epithelial cell density, which suggests cytotoxicity, in ozone-exposed animals 6 h after instillation with endotoxin. This cytotoxic effect appears to be selective for new epithelial cells within the multi-layered, hyperplastic NTE engendered by ozone exposure, because there was no change in the cell density (i.e., decrease) in air-exposed, monolayered NTE of animals instilled with endotoxin at this time point. These new cells may be more sensitive to the cytotoxic effects of endotoxin-induced
inflammation. By 3 days after endotoxin instillation, however, the hyperplasia in ozone/endotoxin-treated animals is increased to levels even greater than that caused by ozone alone.

This sequence of decreased epithelial cellularity (i.e., cytotoxicity) followed by epithelial repair and hyperplasia is reminiscent of the response seen in NTE after ozone exposure. Specifically, after one exposure to ozone, a decrease in cell density occurs concomitantly with the appearance of epithelial neutrophils (Cho et al., 1999b). By 3 days, neutrophil numbers subside while epithelial cell density increases. In the present study, a similar sequence occurred when ozone-exposed animals were instilled with endotoxin. Specifically, cytotoxicity and neutrophil infiltration were evident by 6 h after endotoxin instillation, and both epithelial proliferation and MCM were significantly augmented compared to ozone-exposed, saline-instilled animals. Furthermore, all these endotoxin-induced events were inhibited in neutrophil-depleted animals. That endotoxin, in the absence of neutrophils, was incapable of promoting MCM in this model clearly implicates the neutrophil as a critical mediator of endotoxin's effects. However, our results do not rule out the possibility that, in addition to neutrophils, endotoxin itself or another mediator is required to promote MCM.

We have previously shown that upregulation of mucin gene transcription is an early event after ozone exposure (Cho et al., 1999b). Furthermore we have shown that neutrophils are not required for gene transcription after ozone exposure. Our present study shows that neutrophils are not required for the mucin gene transcription during endotoxin-enhanced MCM by ozone. Specifically, the increase in rMUC 5AC mRNA 6 h after endotoxin treatment in ozone-exposed animals was unaffected by neutrophil depletion. This finding is consistent with our work using nasal explants in vitro, in which endotoxin, in the absence of neutrophils, increases rMuc 5AC mRNA in pre-existing secretory cells

![FIG. 6.](image_url) Effects of ozone, endotoxin, and PMN antiserum on intraepithelial mucosubstances in the NTE of maxilloturbinates. Animals were exposed for 3 days to either filtered air or ozone, followed by treatment with either control serum (neutrophil-sufficient) or PMN antiserum (neutrophil-depleted), and then treated on 2 consecutive days with either saline or endotoxin. Animals were sacrificed either 6 h (A) or 3 days (B) after the last endotoxin instillation, and the volume density (Vs) of mucosubstances in the NTE of maxilloturbinates was determined as described in Materials and Methods. Results are expressed as mean ± SEM; n = 4–6; a, significantly different from respective group exposed to filtered air; b, significantly different from respective group instilled with saline; p ≤ 0.05.

![FIG. 7.](image_url) Effects of ozone, endotoxin, and PMN antiserum on epithelial-cell density in the NTE of maxilloturbinates. Animals were exposed for 3 days to either filtered air or ozone, followed by treatment with either control serum (neutrophil sufficient) or PMN antiserum (neutrophil depleted), and then treated on 2 consecutive days with either saline or endotoxin. Animals were sacrificed either 6 h (A) or 3 days (B) after the last endotoxin instillation, and epithelial cell density in the NTE of maxilloturbinates was enumerated as described in Materials and Methods. Results are expressed as mean ± SEM; n = 4–6; a, significantly different from respective group exposed to filtered air; b, significantly different from respective group instilled with saline; p ≤ 0.05.
proteins. In the absence of neutrophils, the signal for processes responsible for the glycosylation and sulfation of mucin pretreatment of these results is that neutrophils mediate the path-

AB/PAS staining because it lacks reactive groups. One inter-

t февраль группы и sulfated residues within mucous cell globules that containing groups to the core mucin apoprotein. It is these sugar charide groups, and sulfotransferases, which add sulfur-con-

analyze the addition of fucose and sialic acid among other sac-

cider. Taken together, these studies suggest that other mediators produced after ozone or endotoxin exposure are responsible for mucin gene expression, while neutrophils mediate post-transcriptional events that lead to the appearance of intraepithelial mucosubstances.

The protein product of Muc 5AC gene translation undergoes considerable modification by glycosyltransferases, which catalyze the addition of fucose and sialic acid among other saccharide groups, and sulfotransferases, which add sulfur-contain-
ing mucin proteins might not be present. Alternatively, mucin protein may not be translated, despite the transcription of rMuc 5AC. Either possibility requires further study.

In summary, neutrophils mediate the ability of endotoxins to enhance ozone-induced MCM by a mechanism other than upregulation of mucin-gene expression. Our results suggest that neutrophils or neutrophil-derived products mediate an as yet undefined, post-transcriptional event that is necessary to complete metaplastic processes and cause the increased storage of intraepithelial mucosubstances. Although not examined directly in the present study, a number of neutrophil-derived products have been implicated in MCM in airway epithelium, includ-
ing elastase (Voynow et al., 1999), TNF, and IL-1 (Borchers et al., 1999). The role of these and other neutrophil products in endotoxin-enhanced MCM requires further investigation.

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