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ROLE OF NEUTROPHILIC INFLAMMATION IN OZONE-INDUCED EPITHELIAL ALTERATIONS IN THE NASAL AIRWAYS OF RATS

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Hye Youn Cho

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ROLE OF NEUTROPHILIC INFLAMMATION IN OZONE-INDUCED EPITHELIAL ALTERATIONS IN THE NASAL AIRWAYS OF RATS

By

Hye Youn Cho

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ABSTRACT

ROLE OF NEUTROPHILIC INFLAMMATION IN OZONE-INDUCED EPITHELIAL ALTERATIONS IN THE NASAL AIRWAYS OF RATS

By

Hye Youn Cho

Ozone (O_3) is a principal oxidant air pollutant in photochemical smog. More than 50% of the U.S. population live in regions where the atmospheric ozone concentrations exceed the concurrent National Ambient Air Quality Standard for this pollutant. Epithelial cells lining the centriacinar region of lung and the proximal aspects of nasal passage are primary target sites for ozone-induced injury in laboratory animals. Acute exposure of rats to high ambient concentrations of ozone (e.g., 0.5 ppm) results in transient neutrophilic inflammation, epithelial proliferation and mucous cell metaplasia (MCM) in the nasal transitional epithelium (NTE) lining the proximal nasal airways. The principal purpose of the present study was to investigate the role of pre-metaplastic cellular responses, especially neutrophilic inflammation, in the pathogenesis of ozone-induced MCM in the NTE of rats. For this purpose, three specific hypotheses-based whole-animal inhalation studies were conducted. Male F344/N rats were exposed in whole-body inhalation chambers to 0 (filtered air) or 0.5 ppm ozone for 1 - 3 days (8 h/day). Histochemical, immunochemical, molecular and morphometric techniques were used to investigate the ozone-induced cellular and molecular events in the NTE. In addition, two *in vitro* studies, using explants of microdissected maxilloturbinates, were also conducted to examine the effects of ozone-inducible cytokines (*i.e.*, tumor necrosis

factor-alpha; TNF-α, and interleukin-6; IL-6) on mucin gene (rMuc-5AC) expression. Ozone induced a rapid increase of rMuc-5AC mRNA in nasal tissues within hours after the start of exposure. It preceded the appearance of MCM by 3 days, and persisted with MCM for 2 days. Ozone-induced neutrophilic inflammation accompanied the mucin gene upregulation at 1, 2, and 3 days of exposure, but was resolved when MCM first appeared in the NTE at 1 day after 3 days of exposure. Antibody-mediated depletion of circulating neutrophils attenuated ozone-induced MCM, although it did not affect the ozone-induced epithelial hyperplasia and mucin mRNA upregulation. In another study, it was found that pre-existing neutrophilic rhinitis induced by endotoxin augmented the ozone-induced MCM. However, pre-existing rhinitis did not alter the severity of ozone-induced epithelial hyperplasia and mucin gene upregulation. Ozone also induced transient increases in TNF-α and IL-6 mRNA expression concurrently with the initiation of mucin mRNA upregulation in nasal tissues. In addition, exogenous TNF-α and IL-6 induced increases in mucin mRNA levels in microdissected maxilloturbinates *in vitro*, in the absence of neutrophils. In conclusion, ozone-induced MCM is, at least in part, neutrophil-dependent. Though ozone alone is sufficient to induce epithelial proliferation and mucin gene upregulation which are early NTE cell events prior to the development of MCM, neutrophil-mediated inflammatory responses are essential for full phenotypic expression of MCM. Pro-inflammatory cytokines (*i.e.*, TNF-α and IL-6) may be putative mediators of the ozone-induced upregulation of mucin mRNA in the NTE. The results from these series of studies have provided a better understanding of important molecular and cellular mechanisms involved in the pathogenesis of ozone-induced MCM in rat nasal airways.

To my parents
Jung Soo Cho and Moon Ja Lee
for their unconditional love and unending sacrifice

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CHAPTER 1

Introduction

Ozone in the Atmosphere

Ozone (O_3) is a naturally occurring atmospheric gas composed of three atoms of oxygen. It is found both in the stratosphere (atmosphere at altitudes between 20 and 50 km above the earth) and troposphere (atmosphere between the earth's surface and the tropopause, at about 10 to 18 km altitude) of the earth's atmosphere. In the stratosphere, concentration of ozone is high, normally 10 parts per million (ppm), compared to lower concentrations in the troposphere, usually around 20 parts per billion (ppb). The high concentrations of ozone in the stratosphere serve as a natural protective shield for filtering out dangerous levels of ultraviolet radiation from the sun that could cause profound acute and chronic injury to the skin (e.g., sunburn, skin cancer). At high altitudes, ozone is generated by the photochemical reaction of a molecular oxygen with an oxygen atom produced by photodissociation of molecular oxygen by deep ultraviolet radiation (U.S. Environmental Protection Agency (EPA), 1996). Depletion of stratospheric ozone by chlorofluorocarbons generated from refrigerators, solvents or other human activities allows shorter wavelength ultraviolet radiation to be transmitted through the stratosphere and into the troposphere. Therefore, it is currently a major environmental concern and an area of active research. The problems associated with depletion of stratospheric ozone should not, however, be confused with the health and environmental problems associated with elevated levels of ozone in the troposphere where it is a major component of photochemical smog. The primary focus of the present thesis pertains to the health effects of tropospheric ozone and specifically to the potential adverse effects of ambient levels of ozone on the airway epithelial cells lining the upper respiratory tract.

Tropospheric Ozone

Ozone is a highly reactive gas and the principal oxidant pollutant in photochemical smog found in many urban centers throughout the world. It is currently one of the most pervasive problems to human health among the major air pollutants, identified by the U.S. EPA.

To protect human public health, a primary National Ambient Air Quality Standard (NAAQS) for ozone was first designated under the Clean Air Act established by the EPA and passed by the U.S. Congress in 1971. The recently revised NAAQS (July, 1997) states that an ambient air quality monitoring site would be in compliance if the 3-year average of the annual fourth-highest daily maximum 8-hour average ozone concentration does not exceed 0.08 ppm (U.S. EPA, 1997).

It has been estimated by the U.S. EPA that in 1991, 69 million people in the United States lived in areas that were not in compliance with NAAQS for ozone (U.S.EPA, 1991). The episodic high concentrations of ozone, especially during the summer in large urban areas like Los Angeles, where the ambient ozone can reach concentrations as high as 0.2 to 0.3 ppm, poses significant threats to the health of its inhabitants. Even higher ambient levels of ozone have been reported in other metropolitan areas such as Mexico City where 1-h maximum ozone concentrations are often twice as high as those reported in Los Angeles (Calderon Garciduenas *et al.*, 1992, 1995).

The naturally occurring tropospheric ozone is a key intermediate in the degradation of volatile organic compounds (VOCs) emitted in the troposphere from biogenic sources. Though ozone at low concentrations is an important factor in maintaining a clean troposphere, at high concentrations, this irritating compound has the potential to compromise human health. Tropospheric ozone generation is dependent on three principal but complex processes : (1) the emission of nitrogen oxides (NO_x) and VOCs into the atmosphere from anthropogenic and natural sources, (2) the transport of these emissions and their reaction products, and (3) the chemical reactions occurring in the ambient air concurrent with the transport and the emissions (U.S.EPA, 1996).

The principal chemical reaction producing atmospheric ozone in the troposphere as well as in the stratosphere is that between atomic and molecular oxygen (Seinfeld, 1989).

$$O_2 + O + M \rightarrow O_3 + M \qquad (1)$$

where M is any third body (e.g., N_2) that removes the energy of the reaction and stabilizes ozone. In the troposphere, the oxygen atoms are produced by photodissociation of nitrogen dioxide (NO_2):

$$NO_2 + h\nu \rightarrow NO + O \qquad (2)$$

where the photon ($h\nu$) has a wavelength between 280 and 430 nm. The nitric oxide (NO) produced in this reaction reacts rapidly with ozone to regenerate NO_2:

$$NO + O_3 \rightarrow NO_2 + O_2 \qquad (3)$$

The above three reactions occur rapidly, establishing a steady-state ozone concentration according to the photostationary state relation:

$$[O_3] = \frac{J[NO_2]}{k[NO]} \qquad (4)$$

where k is the rate constant for reaction 3 and $J[NO_2]$ is the photolysis rate of NO_2 (reaction 2). Therefore, at a ratio of $[NO_2]/[NO] = 1$, $[O_3]$ predicted by the photostationary state at solar noon in U.S. latitudes is about 20 ppb by volume. Ozone concentrations in unpolluted tropospheric air vary between 20 and 50 ppb. However, in some polluted urban areas, levels as high as 500 ppb (0.5 ppm) are reported (Calderon Garciduenas *et al.*, 1992, 1995).

In polluted urban centers, the steady-state level of ozone is disturbed by the heavy emissions of VOCs as sources of hydrocarbons (RH, where R is an alkyl group) derived mainly from motor vehicles and fuels (Peden *et al.*, 1995). Oxidation of hydrocarbon molecules by hydroxyl radicals produce peroxyradicals (RO_2) that react with NO to form NO_2. Therefore, the one ozone molecule needed to convert NO to NO_2 (reaction 3) is no longer needed in the polluted atmosphere, and this results in a build up of ozone in the ambient air. The net process is :

$$RO_2 + NO \rightarrow NO_2 + RO \qquad (5)$$
$$NO_2 + h\nu \rightarrow NO + O \qquad (6)$$
$$O + O_2 + M \rightarrow O_3 + M \qquad (7)$$

$$\text{Net}: RO_2 + O_2 + h\nu \rightarrow RO + O_3 \qquad (8)$$

Conclusively, the rate of ozone generation is related closely to the rate of RO_2 production in polluted urban areas. The overall process is summarized in Figure 1-1.

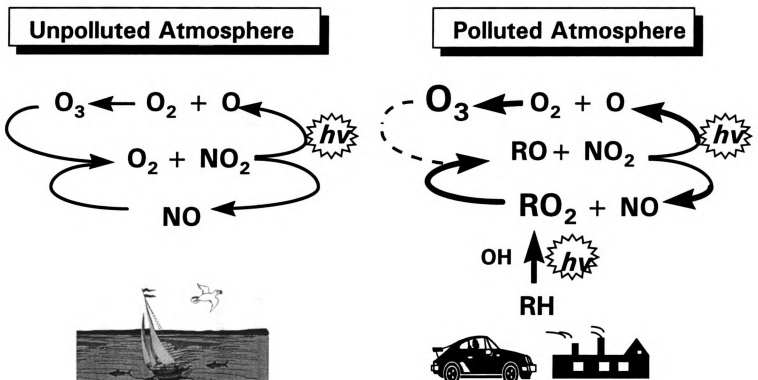


Figure 1-1. Generation of trospheric ozone in unpolluted and polluted atmospheres

Reaction of Ozone in Biologic Systems

Ozone is a potent oxidant. In biological systems, it can react with a variety of macromolecules that are susceptible to electrophilic attack. These include biomolecules containing thiol or amine groups (e.g., proteins) or unsaturated carbon-carbon bonds (e.g., unsaturated fatty acids) (Peden *et al.*, 1995; Pryor, 1992; U.S.EPA, 1996). Because it is such a highly reactive molecule, ozone cannot penetrate the airway lining fluids (mucus and surfactant) and the apical membrane of the surface epithelial cells without reacting with biomolecules present in this air/tissue boundary. Though ozone can directly react with these target molecules, the major effects of ozone in airway tissues must be exerted through toxic products generated from these reactions (Pryor, 1992).

Ozone reaction (ozonolysis) products formed in the body by interaction with lipids or proteins, etc., are a complex array of compounds. Unsaturated fatty acids in the membrane lipid bilayers or in the fluids are known as primary targets for reactions with inhaled ozone. Criegee has proposed that a transient intermediate (carbonyl oxide) is formed during early stage of ozonolysis with the unsaturated fatty acids and that subsequent reactions of this compound determined the final products, depending on the presence or absence of water (Criegee, 1975; Pryor and Church, 1991). In a lipophilic environment, Criegee ozonation leads to the production of ozonides. In the aqueous environment, the carbonyl oxide intermediate generates an aldehyde and a hydroxyhydroperoxide compound, which splits out a hydrogen peroxide to form a second molecule of aldehyde. Figure 1-2 summarizes the chemical reactions of ozone with unsaturated fatty acids.

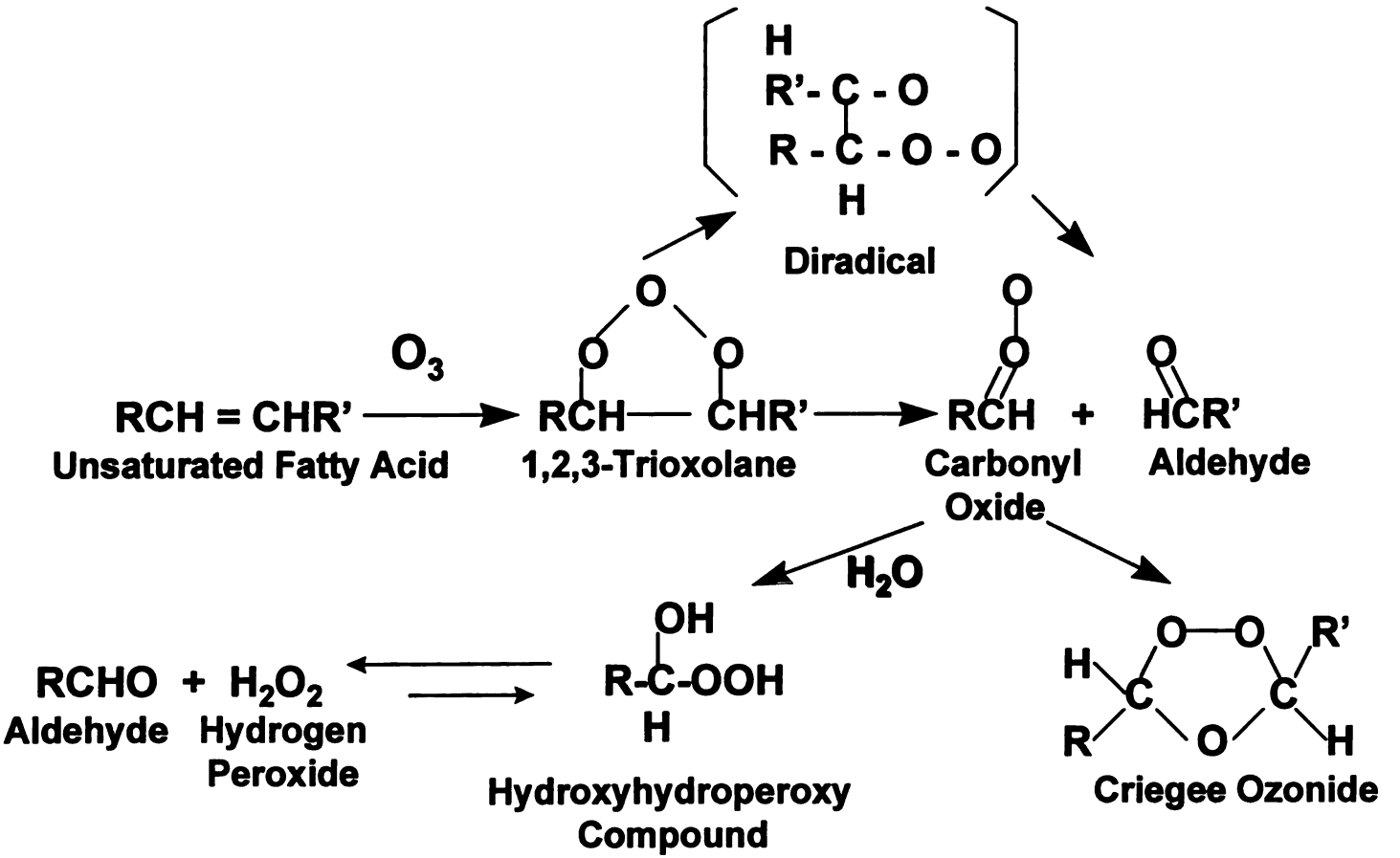


Figure 1-2. Reaction of ozone with unsaturated fatty acid in the presence or absence of water

The ozonolysis products are more stable than ozone itself and able to diffuse further into tissue. These secondary products have the potential to injure resident lung cells such as macrophages and epithelial cells, and could produce tertiary product molecules. Each level of products that is formed in this cascade would have its own characteristic types of biological activity, and relay the effects of ozone increasingly further from the air/tissue barrier potentially resulting in lung damage, inflammation, and changes in host defense capability (Leikauf *et al.*, 1995; Peden *et al.*, 1995).

Human Health Effects of Ozone

Field and Epidemiological Studies

Ozone concentrations at ground level have a wide range of potential adverse effects in respiratory airways of exposed human populations. Recent epidemiological studies addressing the acute effects of ambient ozone have yielded significant associations with health outcomes, including decreases in lung function, aggravation of pre-existing respiratory disease, and increases in daily hospital admissions especially during the most polluted days of the summer (Burnet *et al.*, 1994; Higgins *et al.*, 1990; Spektor *et al.*, 1988, 1991). In most of these studies, the responses of healthy children are similar to those seen in adults.

A number of studies have also addressed the chronic effects of ambient ozone concentrations on the respiratory system. Recent studies of the human health effects in highly air polluted regions of Mexico City have reported significant nasal pathological

changes in the residents. Nasal biopsies from these people often exhibit a wide range of histopathological alterations including marked decreases in the number of ciliated cells, basal cell hyperplasia, squamous cell metaplasia, epithelial dysplasia, submucosal gland proliferation, and mild-to-moderate chronic inflammatory cell infiltration (Calderon Garciduenas *et al.*, 1992, 1995). In epidemiological studies, it is hard to disentangle the effects of ozone from those of other air pollutants (e.g., acid aerosols, particulate matter). Therefore, data from these studies are not enough to definitely conclude that chronic ozone exposure causes significant long-term changes to the human respiratory tract.

Controlled Human Exposure Studies

- Physiological Responses to Ozone Exposure

The physiological responses induced by short-term ozone exposure are separated into respiratory symptoms (e.g., cough, dyspnea), measured lung function responses (e.g., changes in lung volume or airway resistance), and airway responsiveness. In healthy human subjects, the pulmonary responses induced by short-term inhalation exposure to controlled ambient concentrations of ozone (*i.e.*, $\leq$ 0.3 ppm) consist of decreased inspiratory capacity, mild bronchoconstriction, rapid shallow breathing pattern (ozone-induced tachypnea) during exercise, and accompanying symptoms of cough, airway irritation, and chest discomfort associated with deep inspiration (Avol *et al.*, 1983; Folinsbee *et al.*, 1994; Gong, Jr. *et al.*, 1986). Many studies have addressed decrements

in forced expiratory volume (FEV), which reflects decrements in forced vital capacity (FVC) and increases in central and peripheral airway resistance (R_{aw}) in the lungs. Neurogenic inhibition of maximal inspiration by stimulation of C-fiber afferents, either directly or from ozone-induced inflammatory products, is believed to be a possible mechanism leading to the observed pulmonary responses. The responses of healthy children to acute ozone exposure are similar, in most studies, to those seen in adults. Exposure to acute higher concentrations of ozone (0.3 - 0.5 ppm, 2 - 3 h) in exercising or resting healthy adult subjects also induced decrements in the FEV and flows as well as increases in airway responsiveness (Folinsbee *et al.*, 1978; Horvath *et al.*, 1979). The ozone concentration appears to make greater impact on the pulmonary function responses, while mean ventilation and exposure duration serve as secondary determinants of the response at any given ozone concentration (Adams *et al.*, 1981). Similar responses have been seen with prolonged exposures (4 - 8 h) to lower concentrations of ozone (0.08 - 0.16 ppm) (Hazucha *et al.*, 1992). A rapid recovery or attenuation of ozone-induced spirometry (*i.e.*, changes in lung volume) and symptom responses followed the repeated exposure (Hazucha *et al.*, 1992; Horvath *et al.*, 1979). Significant reduction in exercise performance has been also observed in athletes exposed to ozone while they perform high intensity exercise (Schelegle and Adams, 1986; Spektor *et al.*, 1988).

In addition to functional responses, ozone exposure causes airway hyperresponsiveness as demonstrated by an increased physiological response to nonspecific subsequent stimuli such as SO_2 or specific allergens (Golden *et al.*, 1978). It suggests that ozone-exposed airways are predisposed to narrowing of respiratory airways after secondary exposure to a variety of stimuli (Spektor *et al.*, 1988). Changes in airway

responsiveness appear to be resolved but more slowly than are changes in the FEV (Folinsbee *et al.*, 1984). The mechanism underlying the increases of airway responsiveness is only partially understood. Epithelial damages may direct the access of inflammatory mediators (e.g., cytokines, eicosanoids or neuropeptides) to the smooth muscle in airways and result in the increases of airway responsiveness (Hazucha *et al.*, 1996; Kleeberger and Hudak, 1992; O'Byrne *et al.*, 1984).

- Inflammatory Responses to Ozone Exposure

The physiological responses to ozone are accompanied by cellular and biochemical changes in the airways. Short-term exposure to ozone causes acute inflammatory changes throughout the respiratory tract, including the nose. A number of studies have analyzed bronchoalveolar lavage fluid of humans exposed to a single acute ozone (0.2 - 0.6 ppm, 1 - 4 h), which has been used as a useful tool to assess its constitutive elements (e.g., cells, proteins) and the extent and course of inflammation in the lung (Aris *et al.*, 1993; Devlin *et al.*, 1995; Kehrl *et al.*, 1987; Koren *et al.*, 1989a,b; McGee *et al.*, 1990; Schelegle *et al.*, 1991; Seltzer *et al.*, 1986). The analyses of lavage have indicated increases in inflammatory cells (e.g., neutrophils), epithelial cell damage, altered epithelial permeability (*i.e.*, increased serum proteins such as albumin) and production of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6), as well as eicosanoids (e.g., prostaglandin E2). These responses are detectable as early as 1 h after exposure. The 4 - 5-fold increases in the

number of neutrophils by ozone exposure (0.4 - 0.6 ppm) equal or exceed those found in the bronchoalveolar lavage fluid from individuals exposed to other airway irritants (e.g., asbestos or silica) or from individuals with airway disorders such as pulmonary fibrosis or connective tissue disorders (Cherniak *et al.*, 1990). The increased levels of neutrophils and mediators in the lavage fluid persist at least for 18 h after the end of exposure (Koren *et al.*, 1989a,b). The persistent presence of these mediators suggests that they play an important role in resolving inflammation and injury. The time-response profiles vary for different mediators and inflammatory cells (Koren *et al.*, 1991; Schelegle *et al.*, 1991). Another study indicates that these inflammatory responses can occur after acute exposure to lower ambient concentrations of ozone (0.08 - 0.1 ppm, 6.6 h) (Devlin *et al.*, 1991).

Acute ozone exposure causes inflammation and increased permeability even in the nasal passages as indicated by increased levels of neutrophil and albumin in nasal lavage fluid (Bascom *et al.*, 1990; Graham *et al.*, 1988; Graham and Koren, 1990; Henderson *et al.*, 1988; McBride *et al.*, 1994). A recent study in children has presented evidence of a possible relationship between nasal inflammation and measured ambient ozone concentrations (Calderon Garciduenas *et al.*, 1995).

In accordance with clinical data, *in vitro* ozone exposure studies suggest that pulmonary epithelial cells can directly respond to inhaled ozone. Ozone induces production of many mediators (e.g., IL-1β, IL-6, TNF-α, IL-8, prostaglandins) from the epithelial cells as well as from inflammatory cells in culture (e.g., macrophages, neutrophils) (Beck *et al.*, 1994; Devlin *et al.*, 1994; McKinnon *et al.*, 1993).

Exacerbation of Respiratory Disease

People with pre-existing pulmonary disease may be at increased risk from ozone exposure. Because of their existing functional limitations, any further decrease in function would lead to a greater overall functional decline. Furthermore, some individuals with pulmonary disease may have an inherently greater sensitivity to ozone. Therefore, studies of the subpopulation with pre-existing impediments in pulmonary function and exercise capacity are of primary concern in evaluating the health effect of ozone.

Asthmatics, by definition, have inherently increased bronchial responsiveness to inhaled irritants. People with mild to moderate asthma are sensitive to ozone exposure causing further increases (2 - 4-fold) in airway responsiveness (Kreit *et al.*, 1989). In addition, asthmatics exposed to ozone have greater changes in airway resistance and expiratory flow, while they tend to have similar changes in volume-related responses and in symptom responses (e.g., cough and short breath) compared to non-exposed asthmatics (Kreit *et al.*, 1989). McBride *et al.* (1994) have exposed asthmatics with histories of allergic rhinitis to ozone (0.24 ppm, 90 min), and observed significant increases in the numbers of neutrophils and epithelial cells in nasal lavage fluid from the asthmatics compared to those in healthy subjects. This suggests that the upper airways of asthmatic individuals are more sensitive to the acute inflammatory effects of ozone than those of non-asthmatic, healthy subjects. These observations represent a plausible link between elevated ambient ozone concentration during summer and increased hospital admissions for asthmatics. A number of epidemiological studies have shown a consistent

relationship between ambient ozone exposure and acute respiratory morbidity in this population (Krzyzanowski *et al.*, 1992; Lebowitz *et al.*, 1991; Thurston *et al.*, 1997). Especially in children, small decreases in FEV and increases in respiratory symptoms, including exacerbation of asthma, occur with increasing ambient ozone concentration (Koenig *et al.*, 1985).

Increased airway responsiveness to ozone is also reported in subjects with allergic rhinitis (who do not have asthma-like symptoms) but to a lesser degree than that observed in asthmatics (McDonnell *et al.*, 1987). Patients with mild to moderate chronic obstructive pulmonary disease have also shown an alteration in pulmonary responses characterized by decreases in pulmonary functions (e.g., FEV) after ozone exposure (0.12 - 0.41 ppm, 1 - 3 h) at rest or with exercise (Kehrl *et al.*, 1985).

Factors Modifying Responsiveness to Ozone

Factors such as smoking status, age, gender, race, season, and mode of breathing during exposure can also influence the airway responses to ozone (U.S.EPA, 1997). None of these potential influences on the ozone responsiveness has, however, been thoroughly investigated and adequately addressed in clinical studies to date. The observations that healthy older adults appear to be less responsive to ozone than young adults (McDonnell *et al.*, 1993), however, has been confirmed to the point that it can be considered in risk assessment.

Morphological Changes Induced by Ozone in Laboratory Animals

Primary Sites of Injury in the Respiratory Tract

The respiratory tracts in mammalian species are lined by several morphologically distinct epithelial cells (Harkema, 1992; Harkema *et al.*, 1991; Plopper *et al.*, 1983). The epithelial morphology and cell composition vary depending on anatomic regions examined (e.g., nasal airways versus bronchiolar airways). Due to their luminal location, airway epithelial cells form a barrier to the external environment. Therefore, they are the first point of contact for inhaled antigens, particulates or other xenobiotics including ozone, many of which have the potential to cause epithelial cell injury.

Previous studies have documented the injurious effects of ozone on epithelial cells lining the upper and lower respiratory airways in various laboratory animals (Boorman *et al.*, 1980; Plopper *et al.*, 1979). Regional dosimetry and tissue sensitivity are critical factors that determine the distribution of the epithelial lesions caused by ozone exposure in laboratory animals (Kimbell *et al.*, 1993). In these experimental animal models, some of the largest effective doses of inhaled ozone are known to be delivered to two airway regions, the nose and the centriacinar pulmonary region (*i.e.*, the junction of conducting airway and gas exchange region of the lung) (Miller *et al.*, 1985; Overton *et al.*, 1987). In addition, the epithelial cells lining these two district airway regions are believed to be most vulnerable to the toxic effects of ozone (Boorman *et al.*, 1980; Harkema *et al.*, 1987; Plopper *et al.*, 1979).

Centriacinar Region of the Lung

The centriacinar region of the lung, the junction of conducting airway and gas exchange region, is one of the most susceptible airway sites to ozone toxicity. All mammalian species studied react to inhaled ozone ($\leq$ 1.0 ppm) in a similar manner, with species variation in morphological responses depending, in part, on the basic structure of this pulmonary region (e.g., presence or absence of the respiratory bronchioles, the last conducting airways) and the distribution of sensitive cells.

It has been demonstrated that the intensity of the toxicant-induced lesions in centriacinar region is directly related to the acinar volume (Mercer *et al.*, 1991). Acini, the basic structural unit of lung, consist of a terminal bronchus, respiratory bronchioles (when present), alveolar ducts and alveoli. The acini that do not have respiratory bronchioles have a smaller volume than those that do contain these alveolarized bronchioles. In most of the smaller and some of the larger species (e.g., horse, ox, sheep, pig, rabbit, guinea pig, hamster, rat and mouse), there is a single very short, or absent, alveolarized bronchiole that directly joins alveolar ducts (*i.e.*, respiratory bronchioles) (Tyler *et al.*, 1991). In all of these species, the epithelial population is simple cuboidal, with approximately equal numbers of ciliated and nonciliated or Clara cells (Plopper *et al.*, 1983, 1989). Humans and a number of species including monkeys, dogs and cats have centriacinar pulmonary regions characterized by several generations of respiratory bronchioles. Respiratory bronchioles in these have the luminal surface lined by epithelial populations characteristic of more proximal conducting airways, *i.e.*, simple cuboidal epithelium, interrupted by outpoketings lined by Type 1 and Type 2 pneumocytes

characteristic of alveoli in the gas exchange area. Though the precise mechanisms have not been elucidated, the differences in the basic morphology of centriacinar region and size of the acinus seem to contribute to the greater responses of nonhuman primates to ozone (e.g., increases in epithelial thickness and number of cells) compared to those of rodents (Barry *et al.*, 1985; Harkema *et al.*, 1993; Plopper *et al.*, 1991).

Ozone-induced epithelial degenerative changes in the centriacinar regions occur soon after exposure. The epithelial cells most damaged by acute or chronic ozone exposure are ciliated cells and Type I cells (Pino *et al.*, 1992). Both of these cell types have very large surface areas exposed to inhaled gases relative to their cell volume (U.S.EPA, 1996). Loss of cilia and necrosis are characteristic features of injury in ciliated cells after acute ozone exposure (Castleman *et al.*, 1980; Stephens *et al.*, 1974). The type 1 pneumocytes undergo vacuolization, necrosis, and exfoliation leaving bare areas of basement membrane and resistant type 2 pneumocytes after ozone exposure (Stephens *et al.*, 1974). Type 2 cells proliferate and some differentiate into Type 1 cells during the repair process (Evans *et al.*, 1975; Barry *et al.*, 1983). These epithelial changes are accompanied by an acute inflammatory response characterized by increased numbers of neutrophils and alveolar macrophages in the affected centriacinar regions along with hyperemia and interstitial edema, and fibrinous exudate (Boorman *et al.*, 1980; Evans *et al.*, 1975; Fujinaka *et al.*, 1985; Stephens *et al.*, 1974). Repeated, chronic exposures to ozone cause alveolar septal thickening due to (1) increased matrix (*i.e.*, proliferation of fibroblasts and accumulation of collagen) and (2) thickened alveolar epithelium by proliferation of Type 2 cells (Boorman *et al.*, 1980; Fujinaka *et al.*, 1985).

Several studies have examined the postexposure period ("recovery") following acute or chronic ozone exposure. Plopper *et. al.* (1978) reported that the epithelial cells in the centriacinar region of rats returned to normal appearance 6 days after 72 h of exposure, while incomplete resolution has also been reported in various animals species (Ibrahim *et al.*, 1979; Moore and Schwartz, 1981).

Nasal Airways and Mucous Cell Metaplasia

The nose conditions inhaled air and serves as an important defense mechanism ("scrubbing tower") in the upper respiratory tract against many inhaled pollutants (Eccles, 1982; Geurkink, 1983). The "scrubbing" process reduces inhaled airway concentration of ozone and protects the lower respiratory tract from injurious levels of ozone. Since the nose is the port of entry for the respiratory tract, it receives a large dose of inhaled pollutants. It is, therefore, vulnerable to epithelial cell injury caused by a wide range of irritating airborne xenobiotics including ozone, formaldehyde, chlorine and cigarette smoke, and infectious microbial agents (U.S.EPA, 1996).

There is a large range of variation in the structure of the nasal cavity among the laboratory animals as well as between these animal species and humans (Schreider and Raabe, 1981). The complexity of the nasal cavity may affect the inter-species differences in morphological responses to ozone. There is a striking similarity in the nasopharyngeal cavity between nonhuman primates (e.g., macaque monkey) and humans (Schreider and Raabe, 1981). Therefore, studies of the aerosol and gas deposition in this region of the monkey could provide useful information for extrapolation to humans.

Besides differences in architecture of the nose among species, there are also species differences in the distribution of nasal epithelial populations and the type of nasal cells within these populations. There are, however, four distinct nasal epithelial populations in most laboratory animal species (Harkema and Hotchkiss, 1994). They include (1) a stratified squamous epithelium, which is primarily restricted to the nasal vestibule, (2) ciliated, pseudostratified respiratory epithelium in the main nasal chamber and nasopharynx, which consists of six morphologically distinct cell types including numerous mucous (goblet) and ciliated cells, and overlies approximately 46 % of nasal cavity in F344 rats, (3) nonciliated or poorly ciliated transitional epithelium with 1 - 2 cells in thickness, lying between squamous epithelium and respiratory epithelium in the anterior or proximal aspect of the main chamber and consisting of cuboidal, columnar and basal cells with few mucus-producing goblet cells, and (4) the olfactory nerve epithelium located in the dorsal or dorsoposterior aspect of the nasal airways, essential for the sense of smell.

In various laboratory animals, it has been recognized that considerable amounts of inhaled ozone (40 - 70 %) can be absorbed by nasal tissues (Miller *et al.*, 1979; Yokoyama and Frank, 1972). Nasal epithelial cells are known to be principal targets for ozone-induced injury in the upper airways of both rats and monkeys.

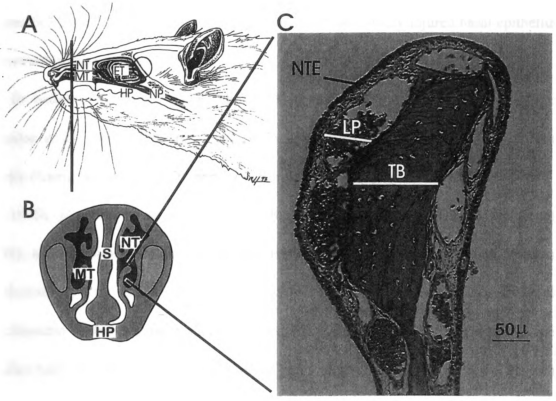
The pathologic effects of ozone on nasal airways of macaque monkeys have been described by Harkema *et al.* (1987a,b). Monkeys exposed to acute or chronic ozone exposures (0.15 or 0.3 ppm, 8 h/day for 6 or 90 days) developed epithelial alterations in the proximal nasal cavity. The lesions were observed mainly in the nasal transitional epithelium (NTE) as well as in the respiratory epithelium lining the anterior nasal

airways. Nasal epithelial lesions were characterized by neutrophilic inflammation, loss of ciliated cells, epithelial hyperplasia, and marked increases of mucous cells and the amount of intraepithelial mucosubstances. An appearance of mucous cells in an epithelium that is normally devoid of the mucous secretory cells (e.g., NTE) is referred to as mucous cell metaplasia (MCM), while an increase in mucous cells in a respiratory epithelium that normally contains some of these secretory cells (e.g., respiratory epithelium) is referred to as mucous cell hyperplasia.

MCM is the principal pathologic change that occurs in the NTE of F344/N rats as well as bonnet monkeys exposed to repeated ozone. Exposure of rats to repeated acute ozone (0.4 - 0.8 ppm, 6 - 8 h/day for $\leq$ 7 days) results in MCM accompanied by epithelial hyperplasia in the NTE (Henderson *et al.*, 1993; Hotchkiss *et al.*, 1991; Reuzel *et al.*, 1990). The nasal lesions including MCM are restricted to the NTE lining the lateral meatus (*i.e.*, maxilloturbinates, lateral sides of nasoturbinates, and lateral walls) in the proximal aspect of the nasal cavity (Figure 1-3). Acute responses of NTE to ozone include epithelial cell death, epithelial cell exfoliation, and neutrophilic influx (Harkema *et al.*, 1989; Hotchkiss and Harkema, 1992; Hotchkiss *et al.*, 1997). NTE cells respond rapidly with DNA synthesis and subsequent cell proliferation prior to the development of MCM (Hotchkiss *et al.*, 1997).

Long-term exposures of rats to ozone (0.5 or 1 ppm for 20 months, or 0.25 ppm for 2 years) have more severe and extensive metaplastic and hyperplastic lesions in the nasal airways (Harkema *et al.*, 1994; Smiler *et al.*, 1988). MCM has also been described in the distal pulmonary airways of rats chronically exposed to high concentrations of ozone (20 months, 1.0 ppm). The MCM in the pulmonary airways, however, was markedly less

Figure 1-3. Anatomic location of the main injury site in the nasal airways of rats exposed to ozone. (A) Exposed lateral wall of nasal airway. MT = maxilloturbinate; NT = nasoturbinate; ET = ethmoturbinate; HP = hard plate; NP = nasopharynx. (B) Anterior face of tissue block from proximal nasal airway. Gray area = nasal tissues including septum (S), MT, NT, and HP; Black area = nasal passage of lateral meatus lined by nasal transitional epithelium (NTE). (C) Digitized image of the MT in this tissue section. NTE lines the luminal surface of the turbinate. TB = turbinate bone; LP = lamina propria containing large blood vessels.


A
NT
MT
HP
HP
B
NT
S
MT
HP
C
NTE
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50μ

severe than that in the NTE of these same rodents (Plopper *et al.*, 1994). No ozone-induced changes have been found in the squamous or olfactory epithelium of rat nasal airways. MCM induced by chronic exposure to ozone (0.5 ppm, for 13 wks) persists for weeks and even months after the end of the exposure, depending on the severity of the initial metaplastic lesions (Harkema *et al.*, 1997). Considering that airway mucus is an efficient anti-oxidant (Cross *et al.*, 1984), it seems likely that development and persistence of the MCM is a protective adaptation of the initially injured nasal epithelium to prevent further damage by ozone.

In addition to ozone, many other inhaled toxicants such as bacterial endotoxin (Gordon *et al.*, 1996; Harkema *et al.*, 1993), sulfur dioxide (Jany *et al.*, 1991), tobacco smoke (Lamb and Reid, 1969), acrolein (Borchers and Leikauf, 1997), chlorine (Wolf *et al.*, 1995), siloxane (Burns-Naas *et al.*, 1998), 3-methylcholanthrene (Rehm and Kelloff, 1991), and machining fluids (Gordon and Harkema, 1995) can induce MCM in airway epithelium of rats. However, little is known about the specific cellular and molecular mechanisms underlying the toxicant-induced MCM in the airway epithelium. Recent studies have investigated the association of inflammatory mediators with the abnormal increases in the production or secretion of airway mucins in laboratory animals. Neutrophil elastase is a well-known mucous secretagogue and induces MCM in rodent airways (Breuer *et al.*, 1985, 1993; Sommerhoff *et al.*, 1990; Jamil *et al.*, 1997; Kim *et al.*, 1987). Both soluble TNF-α and IL-6 induce mucin hypersecretion in airway epithelial cells *in vitro* at concentrations that also cause mucin gene upregulation (Levine

et al., 1994, 1995). Transgenic mice that overexpress IL-4 or IL-5 have MCM (Rankin *et al.*, 1996; Jain-Vora *et al.*, 1997; Lee *et al.*, 1997) and mucin hypersecretion (McBride *et al.*, 1994) with mucin gene upregulation in tracheobronchial or pulmonary airways. However, the precise roles of these inflammatory mediators in the mucous overproduction or hypersecretion are still not clarified.

Mucus in Airways

Function of Mucus

The luminal surfaces of the respiratory, gastrointestinal and reproductive tracts of mammals is covered by a protective mucous layer that is produced and secreted by mucous goblet cells (Figure 1-4) in the surface epithelium and submucosal glands. Mucus is a complex mixture of large glycoproteins (mucins), water, electrolytes, protein, lipid, DNA, and various xenobiotic materials including bacteria and bacterial products (Boat *et al.*, 1976a,b). The mucus hydrates and lubricates the epithelium lining the respiratory tracts from the nasal passages to the respiratory bronchioles in mammals. It serves as an important physical barrier to protect airway tissues against airborne toxicants. Foreign substances entrapped in the luminal mucous layer are constantly removed from the airway by ciliary beating, a process called mucociliary clearance (Rose, 1992; Van Klinken *et al.*, 1995).

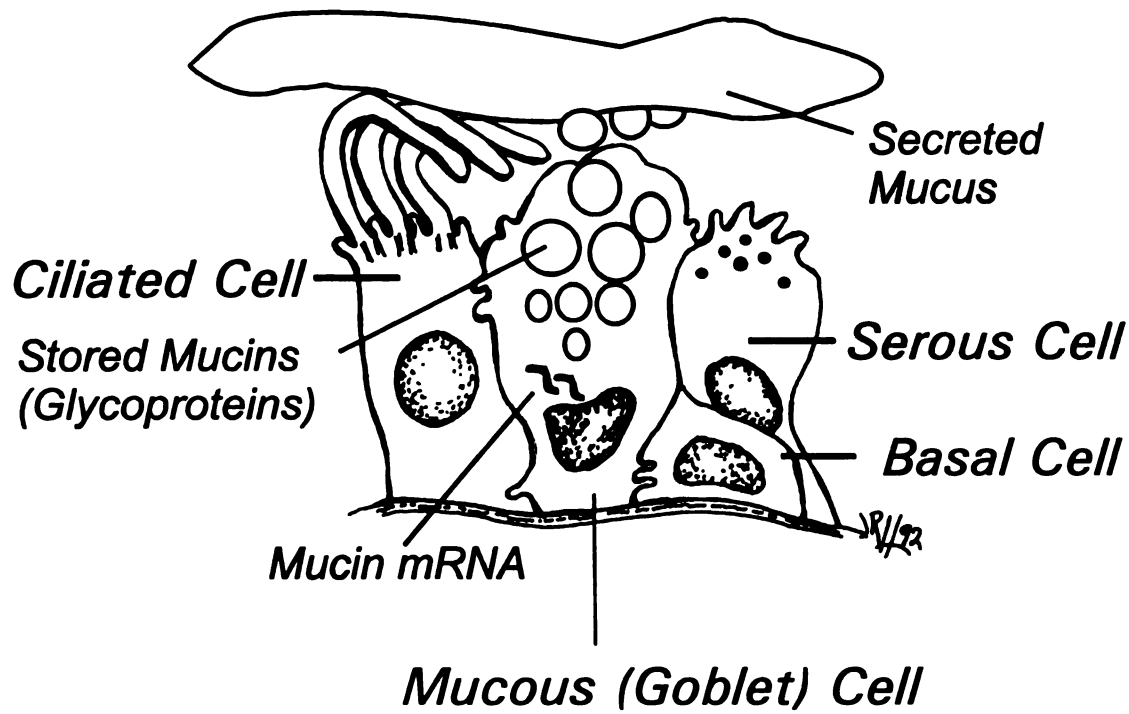

Figure 1-4. The mucous (goblet) cell in airway epithelium responsible for the synthesis, storage, and secretion of mucous glycoproteins (mucins)

Structure and Synthesis of Mucin

The major macromolecular components of mucus are mucins, which are large heterogeneous, high-molecular-weight glycoproteins (200 - 15,000 KD). Due to their large size, high degree of glycosylation, and frequent contamination with non-covalently associated lipids and peptides (Rose, 1992; Slayter *et al.*, 1984), analysis of the primary structure of mucin glycoproteins has been difficult using traditional biochemical and biophysical techniques. Recently, a breakthrough was made by the derivation of antibodies directly to the deglycosylated protein core of mucins by trifluoromethanesulfonic acid- (Edge *et al.*, 1981) or HF-mediated (Shekels *et al.*, 1995) deglycosylation processes. The development of mucin antibodies has led to marked increases in the knowledge of the core protein structures as well as mucin genes by cDNA cloning and sequencing (Perini *et al.*, 1989). To date, nine human mucin genes and encoded mucin proteins (MUC-1, MUC-2, MUC-3, MUC-4, MUC-5AC, MUC-5B, MUC-6, MUC-7 and MUC-8) have been reported (Gendler and Spicer, 1995; Gum, Jr., 1992; Van Klinken *et al.*, 1995), and their homologues have been identified in various animals including rodents (Inatomi *et al.*, 1997; Ohmori *et al.*, 1994; Randell *et al.*, 1996; Shekels *et al.*, 1995). By convention, human mucin genes are designated by MUC, mouse by Muc, and rat by rMuc (Gendler and Spicer, 1995).

Mucins exist as a secretory or a membrane-associated form (Gum, Jr., 1992; Rose, 1992). MUC-1 is the only membrane-associated mucin, identified to date, containing hydrophobic membrane-spanning domains. It is ubiquitously and aberrantly expressed

by various carcinomas, which makes MUC-1 an important marker in malignancy (Braga *et al.*, 1992; Dahiya *et al.*, 1993; Gum, Jr., 1992).

The secretory mucins (except MUC-7) contain two distinct domains : (1) a highly glycosylated core protein region (apomucin), and (2) naked hydrophobic protein flanking region. The central protein core of each mucin molecule contains extended arrays of conserved tandemly repeated peptide sequences (~ 20 amino acid repeat units), which vary between different mucin gene products (Crepin *et al.*, 1990). This apomucin core is rich in serine, threonine and proline. The mucin protein backbone is assembled from the mucin mRNA templates in the rough endoplasmic reticulum (Rose, 1992). As newly synthesized mucin protein moves through the cell from the rough endoplasmic reticulum toward the Golgi apparatus, branched oligosaccharide side chains with 2 - 22 sugars per chain are added to serine or threonine residues in the protein core via O-glycosylation (or rarely N-glycosylation), resulting in 60 - 90 % of mucin mass derived from these carbohydrate side chains. The sugar constituents are N-acetyl-glucosamine, N-acetylgalactosamine, galactose, fucose, and sialic acids. N-acetylgalactosamine is always the initial sugar unit of the oligosaccharide chains transferred to serine or threonine in the mucin core by N-acetylgalactosaminetransferase in the rough endoplasmic reticulum (Rose, 1992). Sequential stepwise O-glycosylation by specific glycosyltransferases leads to formation of core-type structures, which are elongated to completed oligosaccharides. The glycoproteins are then further glycosylated within the Golgi apparatus (specifically in the trans-Golgi compartment) by adding terminal sugar residues such as sialic acids and galactose, or sulfates, which results in the formation of negatively charged mucin glycoproteins (Bennett and Wild, 1991). The extremely diverse composition of sugars

and the degree of sulfation of the oligosaccharide side chains contribute to the inherent heterogeneity of mucin. The high proline contents in the protein core may help to maintain a particular conformation of the apomucin for the close packing of the large carbohydrate side chains.

The 5'- (amino-terminal) and 3'- (carboxy-terminal) flanking regions of mucin protein core contain unique non-repetitive, cysteine-rich sequences. They participate in polymerization of mucin monomers via disulfide bonds to form a high-molecular-weight mucin complex. The timing and process of assembly of mucin subunits into polydisperse macromolecules are not well defined. It has been believed that mucin oligomerization may occur in the endoplasmic reticulum prior to the elongation of oligosaccharide side chains (Dekker and Strous, 1990; McCool *et al.*, 1994). Some core O-glycosylation with the initial sugar unit (*i.e.*, N-acetylgalactosamine) seems to precede the mucin oligomerization to stabilize the extended conformation of mucin peptide so that N- and C-terminal domains are kept well separated. Another line of recent studies has demonstrated that mucin oligomerization takes place downstream to the trans-Golgi compartment with fully glycosylated mucin subunits (Sheehan *et al.*, 1996).

The matured mucous glycoproteins of up to at least decamers are stored in secretory granules (for days in human cells), and then released via periodic exocytosis from the apical surface (Lundgren and Shelhamer, 1990). Once secreted, mucin molecules form a gel via hydrophilic non-covalent bonds between the oligosaccharide of the mucin oligomers to maintain mucous barrier (Strous and Dekker, 1992). MUC-7, a salivary mucin, lacks both a membrane-spanning domain and the cysteine-rich regions, and is the

only soluble secreted mucin identified to date. Figure 1-5 illustrates a hypothetical structure of the secretory mucin monomer.

Tissue-Specificity and Multiplicity of Mucin Expression

Molecular analyses have indicated that expression of secretory mucin is relatively tissue- and cell-specific (Audie *et al.*, 1993; Bobek *et al.*, 1996; Ho *et al.*, 1995; Keates *et al.*, 1997; Shankar *et al.*, 1997, Shekels *et al.*, 1998). In brief, MUC-2 is expressed in the small intestine, colon, and tracheobronchial tissue, and MUC-3 is primarily expressed in small intestine and colon, and gallbladder. MUC-4 is observed primarily in colon and bronchial tissue. MUC-5AC is found in tracheobronchial, gastric tissues, gallbladder and endocervix, and MUC-5B is also found in gallbladder, tracheobronchial tissue and endocervix. MUC-6 is primarily noted in stomach, gallbladder, endocervix, seminal vesicles, pancreas and Brunner's glands. MUC-7 was isolated from salivary glands and MUC-8 encodes a tracheobronchial mucin. However, complete organ distribution studies of these mucins have not been completed.

There are organs such as the lung in which multiple mucin genes or gene products are co-expressed (Aubert *et al.*, 1991; Guzman *et al.*, 1996; Reid *et al.*, 1997; Voynow and Rose, 1994). As indicated above, at least five different mucin genes (MUC-2, MUC-4, MUC-5AC, MUC-5B, and MUC-8) have been detected in respiratory airways. Recent studies have indicated that MUC-5AC as well as its rat homologue (rMuc-5AC) is the major mucin observed in surface epithelium of respiratory airways, while MUC-5B is

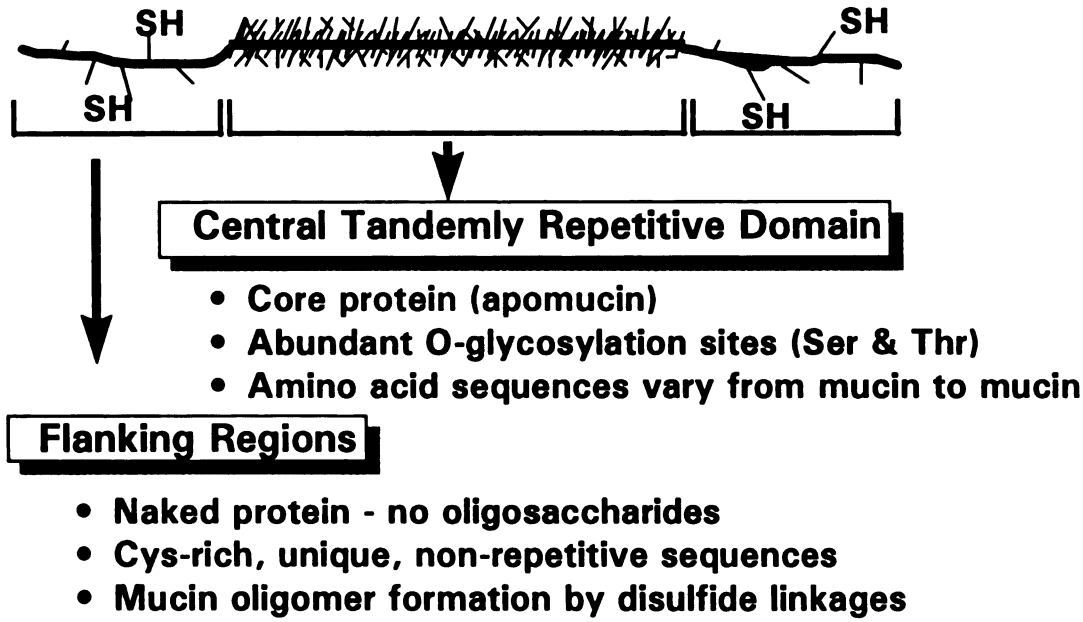

Figure 1-5. Structure of secretory mucin monomer

mainly expressed in submucosal glands (Bluth *et al.*, 1995; Guzman *et al.*, 1996; Hovenberg *et al.*, 1996; Meerzman *et al.*, 1994) . Though no precise link has been made between the mucin gene products observed in airway tissues and mucins present in airway secretion fluids, it is clear that multiple mucins are responsible for the total airway mucin secretion (Shankar *et al.*, 1997; Steiger *et al.*, 1994; Van Klinken *et al.*, 1995).

It is not clear why the body needs several forms of mucin and how tissue-specific regulation of mucin is achieved. Further studies on the structure-function relationship of different mucins might give insights to the multiplicity and tissue-specificity of mucin expression.

Mucin in Pathologic Conditions

Regardless of the beneficial roles of the airway mucus, excess mucus is a frequent problem in respiratory airways. Overproduction and hypersecretion of mucins accompanying MCM are important pathologic features of chronic respiratory diseases such as bronchitis, asthma, rhinitis, and cystic fibrosis (Aikawa *et al.*, 1992; Reid, 1954; Robbins *et al.*, 1984a,b; Chartrard and Marks, 1994). Excess luminal mucus may restrict airflow and plug the conducting airways, and may contribute significantly to the morbidity and mortality associated with these airway chronic respiratory diseases.

In addition to the increased production of mucins, a number of studies have investigated the influence of diseases on the structure of airway mucins. Glycosylation including sulfation of mucins appears to be subject to modification under pathologic conditions (e.g., cystic fibrosis, bronchitis, bronchiectasis). More than 200 types of

oligosaccharide chains have been found in the airway mucus of patients with cystic fibrosis (Lamblin *et al.*, 1991; Rose, 1992). The apparent heterogeneity of mucin oligosaccharide core structure in pathologic tissues may reflect either disease-related alterations in parameters affecting glycosylation and other post-translational modifications (e.g., nucleotide-sugar concentration, altered expression or activity of specific glycosyltransferases, rates of transport of mucin protein through the endoplasmic reticulum and Golgi apparatus), or the activation of specific mucin genes that are more highly expressed in disease states and have different glycosylation patterns.

Regulation of Mucin Gene Expression

Though little is known concerning the underlying mechanisms of accelerated mucin production, increases in steady-state levels of mucin mRNA as a consequence of abnormal control of either transcription rate or RNA stability have been considered as putative regulatory mechanisms of mucin overproduction. Indeed, upregulation of a specific mucin mRNA (e.g., MUC-2) has been frequently reported in patients with cystic fibrosis (Li *et al.*, 1997). Observations from rat models of bronchitis induced by exposure to airway irritants such as ozone, endotoxin, sulfur dioxide, or acrolein, or by viral infections have also shown that the increases in the number of mucous cells and the amount of stored mucosubstances are associated with the elevated level of airway-specific mucin mRNA steady-state (Basbaum *et al.*, 1990; Borchers and Leikauf, 1997; Jany *et al.*, 1991; Li *et al.*, 1997).

Little information has been available on *cis*- and *trans*-acting elements or transcription factors controlling transcription of mucin genes. A recent identification of Nuclear Factor 1 (NF1)-MUC5B and its binding site in the 3' region of MUC-5B gene in differentiated mucous cells has provided a new paradigm with which to examine the transcriptional regulation of mucin gene expression (Pigny *et al.*, 1996). In addition, putative binding sequences for ubiquitous transcriptional factors such as NF-*kB* and Sp1 have been identified in 5'-flanking regions of other mucin genes (*i.e.*, MUC-5AC, MUC-2, rMuc-2) (Li *et al.*, 1998; Nogami *et al.*, 1997). Receptors and downstream signal transduction mechanisms related to the airway mucin gene expression have not been widely studied. However, growth factors such as retinoic acid are well known as positive regulatory factors of mucin gene (MUC-2, MUC-5AC) expression and mucous cell differentiation in airway epithelial cells through the retinoic acid receptor-mediated pathway (Koo *et al.*, 1997). Use of beta-2 receptor agonists indicates that MUC-5AC mRNA upregulation can also occur via activation of G-protein-coupled cell surface receptor pathway in airway epithelial cells (Kherallah *et al.*, 1997). It has been suggested that tyrosine kinase mediates the upregulation of MUC-2 (Li *et al.*, 1997) or MUC-5AC (Voynow *et al.*, 1997) mRNA induced by inflammatory agents (e.g., endotoxin, neutrophil elastase) in cultured epithelial cells.

Mucin mRNA stability may also be involved in the regulation of gene-specific apomucin production. It has been known that several mucin genes (e.g., MUC-2 and MUC-7, rMuc-2) have one or more destabilizing AU-rich elements (AREs) (Van Klinken *et al.*, 1995). Both MUC-5AC and MUC-1 contain different long-tandem-repeats in the 3'-flanking area which are also suggested as other destabilizing sequences (Van Klinken

et al., 1995). Stimulation of the binding of specific nuclear or cytoplasmic proteins to these destabilizing sequences may help to prevent the degradation of mucin mRNA and contributes to the increased steady-state of mucin mRNA levels.

Further elucidation of airway mucin genes and their regulatory mechanisms may aid our understanding of malfunction of mucous-producing goblet cells. In addition, inhibition of regulatory pathways of mucin genes by specific antagonists would constitute a new therapeutic strategy to reduce morbidity and mortality in chronic mucous-obstructive airway diseases such as chronic bronchitis, cystic fibrosis, and asthma.

Specific Aims of Thesis

The goal of the present thesis study was to understand the pathogenesis of ozone-induced nasal epithelial cell responses, hyperplasia and MCM, in the NTE of rats. Specifically, this study was designed to determine the role of neutrophilic inflammation in the cellular and molecular events involved in (1) the proliferation of resistant epithelial cells in the NTE of rats by inhaled ozone, and (2) the metaplastic transformation of nonsecretory epithelium to mucus-secretory epithelium. Based on the previous findings that transient neutrophilic influx is conspicuous and restricted to the NTE where the distinctive ozone-induced epithelial morphological changes occur (Harkema *et al.*, 1989; Hotchkiss *et al.*, 1991, 1997), we have postulated that the infiltrating neutrophils will play important roles in ozone-induced epithelial proliferation and mucin gene upregulation, and ultimately mucous metaplastic transformation in the NTE (Figure 1-6).

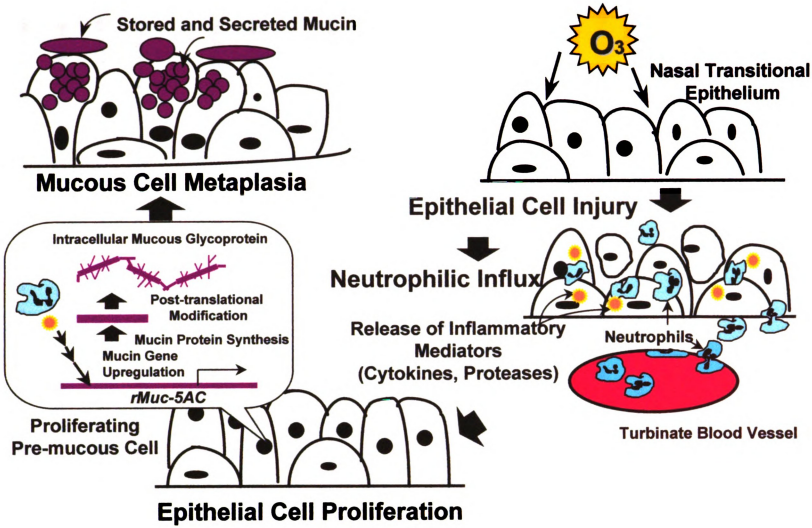

Figure 1-6. Hypothetical pathogenesis of ozone-induced mucous cell metaplasia

Our guiding hypothesis was, therefore, that ozone-induced epithelial hyperplasia and MCM in the rat NTE are mediated by acute neutrophilic inflammation. The specific aims were :

Aim 1: To test the hypotheses that (1) acute ozone exposure induces upregulation of an airway mucin-specific gene (rMuc-5AC) prior to the development of MCM, and (2) neutrophilic inflammation in the NTE precedes or coincided with epithelial DNA synthesis and rMUC-5AC mRNA upregulation.

Aim 2: To test the hypothesis that ozone-induced epithelial proliferation (*i.e.*, DNA synthesis and hyperplasia), mucin gene upregulation and MCM are neutrophil-dependent.

Aim 3: To test the hypothesis that pre-existing neutrophilic rhinitis augments the ozone-induced epithelial hyperplasia, mucin mRNA upregulation and MCM in the NTE.

Aim 4: To test the hypothesis that ozone-inducible pro-inflammatory cytokines (TNF-α, IL-6) cause upregulation of mucin mRNA expression in the NTE.

To address these specific aims, we designed a series of studies using a rat (F344/N, male) model of acute (0.5 ppm, 8 h/day for 3 days) ozone-induced epithelial hyperplasia and MCM. We focused on the ozone-induced alterations occurring in the NTE lining the maxilloturbinates located in the proximal nasal airways of rats (Figure 1-3C in page 23).

We utilized molecular, immunochemical, histochemical, morphometric techniques to characterize mucin mRNA expression, neutrophilic inflammation, epithelial cell injury and proliferation, and MCM in nasal airways. We also employed an *in vitro* tissue culture technique to understand the putative role of individual cytokines on mucin gene expression in the maxilloturbinates.

In the first study, we investigated the effect of acute ozone exposure on nasal mucin mRNA expression, a possible molecular indicator of the following MCM. We also examined the time-dependent changes of ozone-induced inflammatory and epithelial responses in the NTE to determine the correlations between pre-metaplastic responses (*i.e.,* neutrophilic inflammation, rMuc-5AC mRNA expression, epithelial proliferation) and the metaplastic response (MCM).

To determine the contribution of neutrophils in ozone-induced epithelial responses, we depleted rats of circulating blood neutrophils prior to ozone exposure. Systemic administration of a rabbit anti-rat neutrophil antiserum was used for this purpose. We examined the effects of antiserum treatment on the ozone-induced nasal inflammation (*i.e.,* rhinitis), epithelial proliferation, mucin mRNA upregulation, and MCM in the NTE.

To understand further contribution of neutrophilic inflammatory events on the epithelial alterations, we investigated the effect of pre-existing neutrophilic inflammation on the severity of ozone-induced epithelial alterations. For this purpose, we intranasally exposed rats to a strong pro-inflammatory agent, bacterial endotoxin, to induce neutrophilic rhinitis prior to ozone exposure.

In subsequent *in vitro* studies, we investigated the role of soluble mediators (*i.e.*, TNF-α and IL-6), which can be derived from airway cells by ozone exposure, in the mucin gene expression of nasal tissues. First, we examined the time-dependent changes of TNF-α and IL-6 mRNA levels in the nasal tissues of rats exposed to ozone. Then we investigated the effects of the soluble form of these ozone-inducible cytokines on mucin mRNA expressions in explants of microdissected maxilloturbinates, in the absence of neutrophils.

The results of these thesis studies provided new insights into the cellular and molecular mechanisms underlying MCM, a common epithelial alteration in many chronic airway diseases (e.g., chronic bronchitis, asthma, cystic fibrosis and rhinitis) besides the airway alterations induced by air pollutants like ozone.

CHAPTER 2

Inflammatory and Epithelial Responses During the Development of Ozone-Induced Mucous Cell Metaplasia in the Nasal Epithelium of Rats

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Manuscripts submitted to *Toxicological Sciences.*

Abstract

Rats repeatedly exposed to high ambient concentrations of ozone develop mucous cell metaplasia (MCM) in the nasal transitional epithelium (NTE). The present study was designed to determine the temporal relationships of ozone-induced inflammatory and epithelial responses and their correlation with subsequent MCM in the NTE of rats. Male F344/N rats were exposed to 0.5 ppm ozone, 8 h/day for 1, 2, or 3 days. Two h prior to sacrifice, all the rats were injected intraperitoneally with 5'-bromo-2-deoxyuridine (BrdU) to label epithelial cells undergoing DNA synthesis. Rat exposed to ozone for 1 or 2 days were killed 2 h after the exposure. Rats exposed to ozone for 3 days were killed 2 h or 1, 2 or 4 days after the exposure. Control rats were killed after 7-day exposure to filtered air. One nasal passage from the anterior nasal cavity of each rat was fixed and processed for light microscopy to morphometrically determine the numeric cell densities of epithelial cells, neutrophils, and mucous cells, and the amount of intraepithelial mucosubstances in the NTE. The maxilloturbinate from the other nasal passage was processed for analysis of an airway mucin-specific gene (*i.e.*, rMuc-5AC mRNA). Acute ozone exposure induced a rapid increase in rMuc-5AC mRNA levels prior to the onset of MCM, and the increased levels of rMuc-5AC mRNA persisted with MCM. Neutrophilic inflammation coincided with epithelial DNA synthesis and upregulation of rMuc-5AC, but was resolved when MCM first appeared in the NTE. The results of the present study suggest that upregulation of mucin mRNA by acute ozone exposure are associated with the concurrent neutrophilic inflammation and epithelial hyperplasia in the NTE, and that

ozone-induced MCM may be dependent on these important pre-metaplastic responses (*i.e.*, mucin mRNA upregulation, neutrophilic inflammation and epithelial proliferation).

Introduction

Ozone (O_3) is an irritating oxidant gas in photochemical smog, and one of the regulated criteria air pollutants for which National Air Quality Standards have been designated under the Clean Air Act (Steinfeld, 1991). Controlled inhalation studies have demonstrated that acute ozone exposure induces cellular and biochemical changes in the pulmonary airways of human subjects (Schelegle *et al.*, 1991; Seltzer *et al.*, 1986). Ozone-induced morphologic changes in the distal centriacinar regions of the lung in laboratory animals have been well documented in numerous studies (Castleman *et al.*, 1980; Dungworth, 1989; Stephens *et al.*, 1974).

The airways of the upper respiratory tract, specifically the nose, are also susceptible to ozone toxicity. Nasal inflammation has been induced in human volunteers acutely exposed to high ambient concentrations (0.4 - 0.5 ppm for 2 - 4 h) of ozone (Graham *et al.*, 1988; McBride *et al.*, 1994; Bascom *et al.*, 1990). In addition, nasal epithelial lesions thought to be related to exposure to air pollution have been described in people living in ozone-polluted atmospheres of southwest metropolitan Mexico City (Calderon Garciduenas *et al.*, 1992, 1995). Marked inflammatory and epithelial responses to near ambient concentrations of ozone have also been demonstrated in the nasal mucosa of both monkeys and rats (Harkema *et al*, 1987a,b, 1993; Hotchkiss *et al.*, 1989).

We have previously reported that acute or chronic exposures of 0.5 - 1.0 ppm ozone causes epithelial proliferation and marked mucous cell metaplasia (MCM) in surface epithelium lining the lateral meatus of the proximal nasal airways (*i.e.*, nasal transitional epithelium; NTE) in F344/N rats (Harkema *et al.*, 1989, 1992, 1997; Hotchkiss *et al.*, 1991). The ozone-induced MCM in rat nasal epithelium was similar in character to nasal epithelial changes previously reported by Harkema *et al.* (1987b) in macaque monkeys repeatedly exposed to 0.15 or 0.3 ppm ozone for 6 days or 13 wks (6 h/day, 5 days/wk).

Though the ozone-induced morphological changes in the nasal epithelium have been well characterized, the cellular and molecular events preceding the onset of MCM and epithelial hyperplasia have not been thoroughly investigated. Previous studies have demonstrated that a transient neutrophilic inflammation is conspicuous in the nasal epithelium prior to the development of MCM (Hotchkiss *et al.*, 1989, 1997). However, little is known about the relationship of the neutrophilic inflammation with the ozone-induced epithelial alterations in the nasal airway. In addition, the effects of ozone exposure on mucin gene expression as well as its relationship to ozone-induced MCM in the NTE have not been previously investigated. To understand further how and when ozone induces nasal cell injury and reparative and adaptive changes (*i.e.*, epithelial proliferation and MCM) in the NTE of rats, it is first important to determine clearly the temporal relationship of ozone-induced epithelial and inflammatory responses that occur early after the start of exposure and during the development of the mucous metaplastic changes.

Therefore, the present study was designed to test the hypotheses that (1) acute ozone exposure induces upregulation of mucin gene expression prior to the development of

MCM, and (2) neutrophilic inflammation precedes, or is concurrent with, mucin gene overexpression and other pre-metaplastic events (e.g., hyperplasia). For this purpose, rats were exposed to 0.5 ppm ozone for 1 - 3 days (8 h/day). Some of the 3-day-exposed rats were held in air for an additional 1 - 4 days. We determined the time-dependent inflammatory and epithelial cell responses in the nasal epithelium of the ozone-exposed rats. We also determined the temporal expression of rMuc-5AC mRNA in the nasal tissues during and after ozone exposure. In addition, the temporal relationship of mucin gene upregulation with (1) neutrophilic inflammation, (2) epithelial proliferation (*i.e.*, DNA synthesis and numeric density), and (3) onset of MCM was investigated. A better understanding of these exposure-related cellular and molecular events provides new insights into the pathogenesis of MCM caused by repeated ozone exposure.

Materials and Methods

Animals and exposure. Fifty-six male F344/N rats (Harlan Sprague-Dawley, Indianapolis, IN), 10 - 12 weeks of age, were randomly assigned to one of 7 exposure groups (n = 8/group). Male rats were chosen for all experiments to avoid hormonal changes during the estrous cycle. Estrous cycle-related changes have been shown to alter secretory cell proliferation in rodent airways (Hayashi *et al.*, 1979).

Rats were housed two per cage in polycarbonate shoebox-type cages with Cell-Sorb Plus bedding (A&W Products, Inc., Cincinnati, OH) and filter caps. Water and food (Tek Lab 1640; Harlan Sprague Dawley, Indianapolis, IN) were available *ad libitum*. The rats were maintained on a 12-h light/dark cycle beginning at 6:00 a.m. under controlled temperature (16 - 25°C) and humidity (40 - 70%).

Prior to the start of the inhalation exposure, rats were conditioned in whole-body exposure chambers (HC-1000, Lab Products, Maywood, NJ) supplied with filtered air for 1 day. The rats were individually housed in rack-mounted stainless-steel wire cages with free access to food and water prior to exposure. The chamber temperature and relative humidity as well as room light setting were maintained as described above.

Rats in one exposure group were exposed to 0 ppm ozone (filtered air) for 7 days (controls, n = 8). Rats in the other seven exposure groups (n = 8/group) were exposed to 0.5 ppm ozone, 8 h/day, for 1, 2, or 3 days. The rats were exposed to ozone or filtered air in the whole-body exposure chambers from 6 am to 2 pm in the Inhalation Toxicology Exposure Laboratory housed in the University Research Containment Facility at

Michigan State University. Though food was removed, animals had free access to water during the exposure. Ozone was generated with an OREC Model O3VI-O ozonizer (Ozone Research and Equipment Corp., Phoenix, AZ) using compressed air (AGA Gas, Lansing, MI) as a source of oxygen. No NO_x gases have been detected by this method of generation which uses U.V. light to convert oxygen to ozone (Sun *et al.*, 1988). Dilution air was mixed with ozone and delivered to the chambers using teflon tubing. The total airflow through the exposure chambers was maintained at approximately 250 L/min (15 chamber air changes/h). The chamber temperature and relative humidity during the exposure remained the same as those during the animal conditioning period. The chamber ozone concentration was controlled by adjusting the intensity of U.V. radiation within the ozonizer. It was monitored throughout the exposure with Dasibi 1003 AH ozone monitors (Dasibi Environment Corp., Glendale, CA), and recorded by Linear 0141 strip chart recorders (Linear Instrument Corp., Reno, NV). The exposure-atmosphere sampling probes were positioned in the breathing zone of the rats within the middle cage rack of the HC-1000 chambers. The chamber ozone concentrations (mean $\pm$ standard deviation) during the 3-day exposure to 0.5 ppm-ozone were 0.523 ± 0.006. The chamber ozone concentrations during 7-day exposures to filtered air were maintained less than 0.05 ppm.

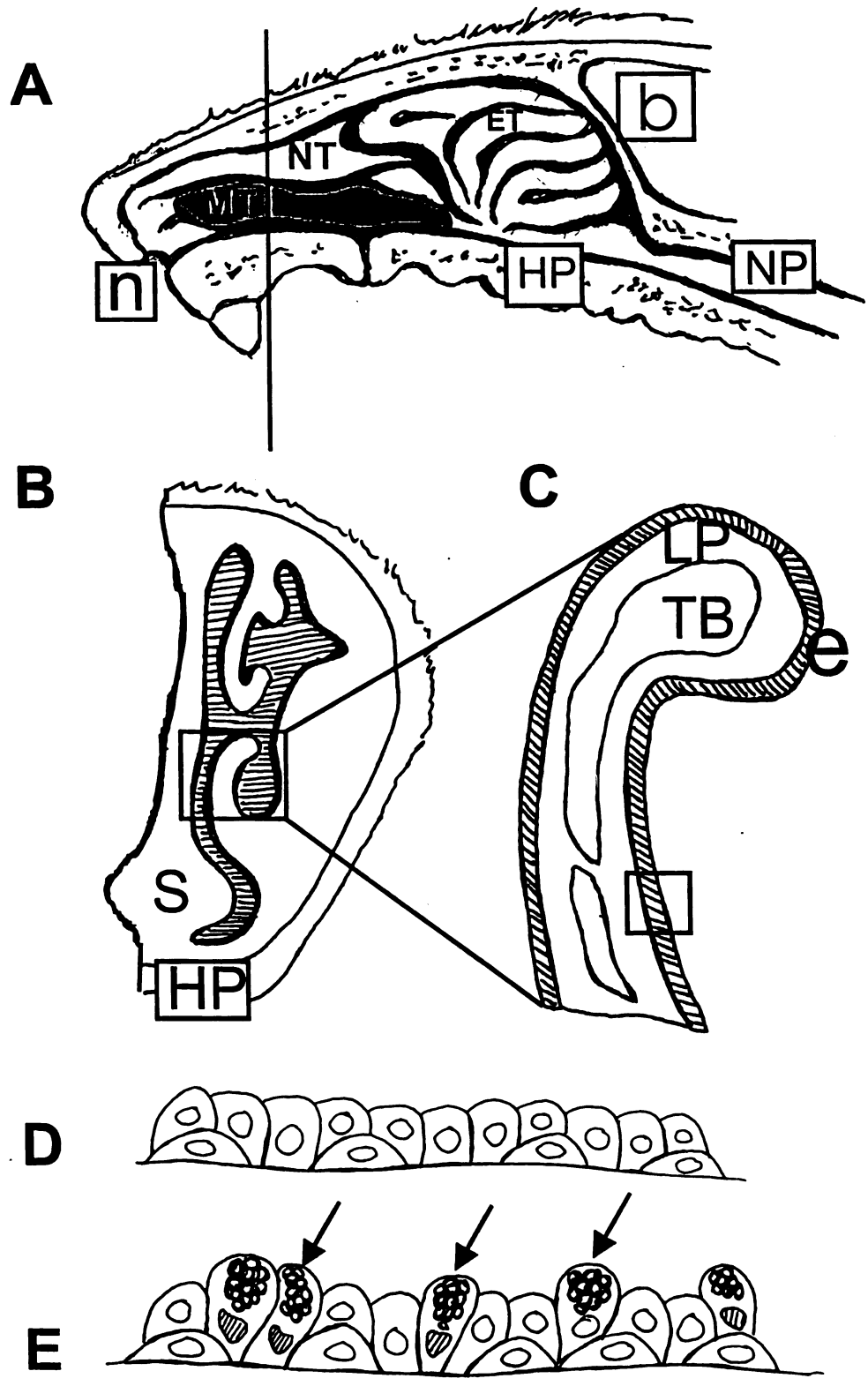
Tissue selection and preparation for analyses. Two hours prior to the designated sacrifice, each rat was treated intraperitoneally (*ip*) with 5'-bromo-2-deoxyuridine (BrdU; 50 mg/Kg body wt.) to label cells undergoing DNA synthesis in the S-phase of the cell cycle. The rats exposed to ozone for 1 or 2 days were killed 2 h after the end of exposure.

The rats exposed to ozone for 3 days were killed 2 h or 1, 2, or 4 days after the end of exposure. Control rats were sacrificed after 7 days of exposure to filtered air. Rats were deeply anesthetized using 4% halothane in oxygen and killed by exsanguination via the abdominal aorta.

Immediately after death, the head of each rat was removed from the carcass. After the eyes, lower jaw, skin and musculature were removed from head, the nasal airways were opened by splitting the nose in a sagittal plane adjacent to the midline. The maxilloturbinate (Figure 2-1A) from one nasal passage was excised by microdissection and immediately homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH). The homogenate was snap frozen in liquid nitrogen and stored at -80°C until processed for isolation of total RNA and analysis of mucin mRNA.

The other nasal passage was immersed in a large volume of zinc formalin (Anatech, Ltd., Battle Creek, MI) for at least 24 h. The zinc formalin-fixed nasal tissues were decalcified in 13% formic acid for 4 days, and then rinsed in tap water at least 2 h as previously described by Harkema *et al.* (1988). A tissue block was removed from the proximal aspect of the nasal cavity by making two transverse cuts perpendicular to the hard plate. The first cut was immediately posterior to the upper incisor tooth (Figure 2-1A), and the second cut was at the level of the incisive papilla. The tissue block was excised, embedded in paraffin, and 5 μm-thick sections were cut from the anterior face of the tissue block. One nasal tissue section from each animal was histochemically stained with hematoxylin and eosin for morphological identification of epithelial cells. Another tissue section from each animal was immunohistochemically stained with anti-BrdU

Figure 2-1. Anatomic location of nasal tissues selected for morphometric and RT-PCR analyses. (A) Exposed lateral wall of nasal airway. Shaded area indicates the maxilloturbinate (MT) in a nasal passage microdissected for mucin mRNA analysis. The vertical line indicates anterior surface of the transverse block used for morphometric analysis. n = naris; NT = nasoturbinate; ET = ethmoturbinate; HP = hard plate; NP = nasopharynx; b = brain. (B) Anterior face of tissue block from one proximal nasal airway. S = nasal septum. (C) Enlarged views of the maxilloturbinate in B illustrating the major turbinate tissue compartments, TB = turbinate bone; LP = lamina propria; e = surface epithelium (NTE). (D) Enlarged view of the NTE lining the maxilloturbinates of a normal (control) rat. The NTE is a nonciliated cuboidal epithelium, 1 - 2 cell layers thick, with no mucous secretory cells. (E) Enlarged view of ozone-exposed NTE with ozone-induced MCM. Note the appearance of numerous mucous cells (arrows) within the exposed epithelium.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antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) to detect BrdU-labeled nuclei (Johnson *et al.*, 1990) (Figure 2-2), and counterstained with hematoxylin (Gill 3; Ricca Chem. Co., Arlington, TX). A third tissue section from the same block was stained with Alcian Blue (pH 2.5)/Periodic Acid-Schiff's sequence (AB/PAS) to identify acidic and neutral mucosubstances in the surface epithelium.

Morphometry of neutrophilic inflammation, epithelial cell numeric density and DNA synthesis. The NTE lining the maxilloturbinate of each animal was analyzed using computerized image analysis and standard morphometric techniques (Hotchkiss and Harkema, 1992; Hotchkiss *et al.*, 1991). The neutrophil numeric density was determined by quantitating the number of nuclear profiles of neutrophils in the surface epithelium lining the maxilloturbinates (*i.e.*, NTE), and dividing the number by the total length of the basal lamina underlying this epithelium (*i.e.*, intraepithelial neutrophils/mm basal lamina). Neutrophils were identified by morphologic characteristics that included their size, darkly stained multi-lobed nucleus, and clear cytoplasm with dust-like granules. The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina using a Power Macintosh 7100/66 computer and the public domain image analysis software (NIH Image; written by Wayne Rasband at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The epithelial cell numeric density (*i.e.*, epithelial nuclei/mm of basal lamina) was determined by counting the total number of epithelial cell nuclear profiles present in the NTE covering the maxilloturbinate, and dividing this

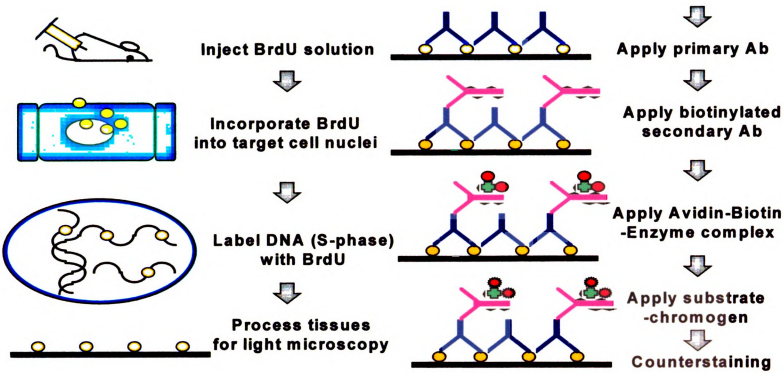

Figure 2-2. Immunostaing of BrdU using avidin-biotin complex method to determine cells undergoing DNA synthesis in the S-pahse of the cell cycle

number by the length of basal lamina. The epithelial cell labeling index (LI) was determined as an indicator of the epithelial DNA synthesis. The number of BrdU-labeled NTE cell nuclei was counted, divided by the total number of epithelial cell nuclei and multiplied by 100 (*i.e.*, % BrdU-labeled epithelial cell nuclei).

Morphometry of stored intraepithelial mucosubstances and mucous cells. To estimate the amount of the intraepithelial mucosubstances in NTE lining the maxilloturbinates, the volume density (Vs) of AB/PAS-stained mucosubstances was quantified using computerized image analysis and standard morphometric techniques. The area of the AB/PAS-stained intraepithelial mucosubstances was calculated by the image analysis software program from the automatically circumscribed perimeter of the stained material. The length of the basal lamina underlying the surface epithelium was determined as described above. The volume of stored mucosubstances per unit of surface area of epithelial basal lamina was estimated using the method previously described in detail by Harkema *et al.* (1989, 1987b), and expressed as nL/mm^2 basal lamina.

The numeric cell densities of mucous cells (epithelial cells containing AB/PAS-stained mucosubstances) in the NTE lining the maxilloturbinates were also morphometrically determined. Only AB/PAS-positive epithelial cells with a nuclear profile were counted, and the data were expressed as the number of mucous cell nuclei/mm of basal lamina.

Analysis for mucin mRNA in maxilloturbinates. Total cellular RNA was isolated from the maxilloturbinate homogenate according to the method of Chomczynski *et al.* as outlined in the Tri-reagent product literature (Chomczynski and Sacchi, 1987). To avoid DNA contamination, the isolated RNA pellet was resuspended in nuclease-free water and treated with 10 units RNase-free DNase I (Boehringer Mannheim GmbH, Germany) in 5X Transcription buffer (Promega, Madison, WI) at 37°C for 30 min. The RNA was extracted sequentially with equal volumes of phenol/chloroform/isoamyl alcohol mixture (25:24:1) and chloroform/isoamyl alcohol mixture (24:1), and precipitated. The final pellet was washed with 75% ethanol, air dried, resuspended in nuclease-free water containing rRNasin (40 units/100 μl), and the concentration of RNA was determined by measuring absorbances at 260 nm. All the RNA samples were stored at -80°C.

The RNA was analyzed to determine the steady-state levels of rMuc-5AC mRNA by quantitating the amount of rMuc-5AC cDNA produced by reverse transcriptase polymerase chain reaction (RT-PCR). Cyclophilin is an abundant and ubiquitous cellular protein well known as a major intracellular receptor for the immunosuppresant cyclosporin A, and considered as a putative molecular chaperone (Matouschek *et al.*, 1995; Kern *et al.*, 1994). Because cyclophilin mRNA expression was similar in all experimental groups, this housekeeping gene was used as an internal standard in this semi-quantitative RT-PCR analysis.

Primers specific for rat rMuc-5AC cDNA and all-species cyclophilin cDNA sequences were synthesized and purified by the Macromolecular Structure Facility at Michigan State University. The sequences of the forward and reverse primers for

cyclophilin are 5'- CTT GTC CAT GGC AAA TGC TG-3' and 5'-GTG ATC TTC TTG CTG GTC TTG-3', respectively. The sequences of the rat Muc-5AC forward and reverse primers are 5'-CAT CAT TCC TGT AGC AGT AGT GAG G-3' and 5'-GGT ACC CAG GTC TAC ACC TAC TCC G-3', respectively. The predicted amplified sizes of the cyclophilin cDNA and rMuc-5AC cDNA products were ~190 bp and ~320 bp, respectively.

A 50 ng/μl working solution of each RNA sample was prepared, and RT-PCR was performed in 9600 Perkin Elmer Thermocycler. Two μl (100 ng) aliquots were reverse transcribed into cDNA in a volume of 20 μl, each, containing PCR buffer (166 mM $(NH_4)_2SO_4$, 50 mM β-mercaptoethanol, 67 μM EDTA, 0.67 M Tris, pH 8.8, 0.8 mg/ml BSA) plus 5 mM $MgCl_2$, 1 mM each dNTP, 10 units rRNasin, 125 ng oligo$(dT)_{15}$ and 50 units of Moloney Murine Leukemia Virus reverse transcriptase (M-MLV- RT; Gibco BRL, Gaithersburg, MD). A PCR master mix consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmol of each forward and reverse primers of MUC-5 mRNA and cyclophilin mRNA, and 1.25 units Taq DNA polymerase was added to each cDNA sample for a final volume of 50 μl. The Taq was added after the PCR master mix was heated to 85°C for 5 min to minimize primer dimer formation. PCR amplification was started with 3 min incubation at 95°C followed by a three step temperature cycle; denaturation at 95°C for 30 sec, annealing at 56°C for 1 min and extension at 72°C for 1 min for 25 cycles. A final extension step at 72°C for 10 min was included after the final cycle to complete polymerization. The number of cycles was chosen to ensure that amplification of cyclophilin (most abundant gene) did not reach a plateau level.

The abundance of mRNA was semi-quantitatively determined by densitometric analysis of ethidium bromide-stained agarose gel (3%, Nusieve:agarose = 3:1) using the Gel Doc 1000 analysis system (BioRad Laboratories, Inc., Herculese, CA) and Molecular Analyst Software Version 2.1 on a Power Macintosh 7100/80. The volume of the rMuc-5AC cDNA band was divided by the volume of the cyclophilin cDNA band. To minimize the variability of analysis, the quantitation for all the samples was performed at the same time.

Statistical analyses. All data were expressed as the mean group value ± the standard error of the mean (SEM). The natural logarithms of the data were used for statistical analysis to make the variances approximately equal for all groups. The data were first analyzed using one-way analysis of variance (ANOVA) to identify the time-response of inflammation and epithelial events induced by ozone exposure. Significant differences between air control group and ozone-exposed groups were evaluated using Dunnett's Method. Statistical analyses were performed using a commercial statistical analysis package (SigmaStat; Jandel Scientific Software, San Rafael, CA). The level of statistical significance was set at $p \leq 0.05$.

Results

Histopathology of nasal mucosa

Exposure-related nasal lesions were present only in rats exposed to 0.5 ppm ozone. No lesions were observed in the nasal airways of filtered air (0 ppm ozone)-exposed rats (controls). In all of the rats exposed to 0.5 ppm ozone, regardless of the duration of the exposure, the nasal lesions were restricted to the mucosa containing the NTE that lined the lateral meatus in the proximal nasal cavity. The character and severity of these site-specific mucosal lesions, however, varied according to the number of ozone exposures and the length of time post-exposure. Figure 2-3 contains light photomicrographs of representative maxilloturbinates that depict time-dependent changes of inflammatory and epithelial cell responses in the NTE. Time-dependent progression of MCM in the NTE is illustrated in Figure 2-4 that contains representative maxilloturbinates stained with AB/PAS for identification of intraepithelially stored mucosubstances.

After one day of exposure to 0.5 ppm ozone, the principal morphologic alterations in the NTE were epithelial degeneration and atrophy due to individual cell necrosis and exfoliation. These ozone-induced alterations in the NTE were most noticeable in the dorsal and medial aspect of the maxilloturbinate, the lateral ridge of the nasoturbinate and the dorsal recess of the lateral wall. Concurrent with the epithelial lesions was a mild-moderate inflammatory response in the affected mucosa, characterized by endothelial margination of neutrophils in the large capacitance vessels of the lamina propria and an

influx of neutrophils in the adjacent interstitial tissue of the lamina propria and also extending into the NTE.

After 2 days of ozone exposure, the neutrophilic inflammatory response in the nasal mucosa was similar to that observed after one day of exposure to ozone with conspicuous accumulations of neutrophils in both the lamina propria and NTE. However, the epithelial degeneration and atrophy of the NTE observed in the one day-exposed rats was replaced by a regenerative, basophilic epithelium in the rats exposed to ozone for 2 consecutive days. The principal features of the regenerative NTE were mild hyperplasia of basal cells and the appearance of widely scattered mitotic figures. In addition, necrotic epithelial cells, a common feature in the NTE of the one day-ozone exposed animals, were infrequently observed in the NTE of the 2-day exposed rats.

Epithelial hyperplasia was the principal feature in the NTE of rats exposed for 3 consecutive days and sacrificed at the end of the exposure. The hyperplastic NTE in these ozone-exposed rats was approximately 3 - 4 cells in thickness compared to 1 - 2 cells in the NTE of control rats exposed only to filtered air. Only a mild influx of neutrophils was present in the nasal mucosa containing hyperplastic NTE after 3 days of ozone exposure.

In rats exposed to 0.5 ppm ozone for 3 days and sacrificed one day after the end of the exposure, the NTE remained hyperplastic but there were also a few widely scattered AB/PAS-stained, mucous cells in the NTE indicating the onset of a mild MCM. Only a few widely scattered neutrophils were present in the NTE or lamina propria of these repeatedly exposed rats.

Ozone-induced MCM in the NTE was even more marked in rats exposed to 0.5 ppm ozone for 3 days and killed 2 or 4 days postexposure. The 3-day-ozone-exposed rats that were killed 4 days after the end of the exposure had the most severe MCM compared to the other 3-day-ozone-exposed rats killed 1 or 2 days postexposure. Epithelial hyperplasia was also a conspicuous feature of the NTE in rats killed 2 or 4 days postexposure. However, no associated inflammatory cell influx was present in the nasal mucosa with the hyperplastic and metaplastic NTE of the 3-day-ozone-exposed rats killed 2 or 4 days after the end of the exposure.

Morphometric quantitation

Neutrophilic influx. Exposure to 0.5 ppm ozone resulted in a transient influx of neutrophils within the NTE lining the maxilloturbinates (Figure 2-5). Significant increases in the number of intraepithelial neutrophils were evident in rats exposed to 0.5 ppm ozone for 1 and 2 days (38- and 47-times greater than air-exposed controls, respectively). The number of neutrophils in the NTE was attenuated (~ 45%) in rats exposed to ozone for 3 days, compared to those exposed to ozone for 1 or 2 days. At one day after 3 days of ozone exposure, the number of intraepithelial neutrophils was 10-fold greater than air-exposed controls. However, the intraepithelial neutrophil numbers returned to control levels in rats killed 2 and 4 days postexposure.

Epithelial cell DNA synthesis. Only a few widely scattered BrdU-labeled NTE cells were present in air-exposed control rats (Figure 2-6). There was a marked, but transient, increase in the number of BrdU-labeled cells in rats exposed to ozone for 2 and 3 days, with 112-fold and 143-fold increases, compared to the controls, respectively. The numbers of BrdU-labeled cells in rats exposed to 0.5 ppm ozone and killed 1, 2, or 4 days postexposure were similar to those of air-exposed control rats.

NTE cell numeric density. Ozone exposure altered the number of NTE cells lining the maxilloturbinates (Figure 2-7). Rats exposed to ozone for 1 day had 9% fewer NTE cells than air-exposed control rats. However, rats exposed to ozone for 2 days had NTE cell numeric densities that were not significantly different from those of controls. After 3 days of ozone exposure, rats had significantly more NTE cells (36%), compared to air-exposed controls. This ozone-induced NTE cell hyperplasia persisted in rats killed 2 and 4 days postexposure (*i.e.*, 52% and 37% more cells than those in controls, respectively).

Amount of stored intraepithelial mucosubstance. Little AB/PAS-stained intraepithelial mucosubstances were present in the NTE of control rats or rats exposed to ozone for 1 or 2 days (Figure 2-8). An ozone-induced increase in the amount of stored mucosubstances was first detected in the NTE of rats exposed to ozone for 3 days and sacrificed 2 h later. A significant increase (24-fold, compared to controls) in the amount of the intraepithelial mucosubstances was observed in rats sacrificed 1 day after the 3-day-ozone exposure. The amount of intraepithelial mucosubstances was increased with time postexposure.

Thirty and fifty fold more Vs, compared to that of controls, were observed in rats killed 2 and 4 days postexposure, respectively.

Mucous cell numeric density. Control animals exposed to filtered air (0 ppm ozone) had no or only a few mucous cells in the NTE. A significant increase (22-fold more than air-exposed controls) in the number of mucous cells was first detected in 3-day-ozone-exposed rats killed 1 day postexposure (Figure 2-9). The numeric density of mucous cells in the NTE of ozone-exposed animals increased with time postexposure. Rats killed 4 days after the 3-day-ozone exposure had 43-fold more mucous cells than air controls.

Mucin mRNA (rMuc-5AC) abundance

Figure 2-10 depicts an agarose gel with representative RT-PCR cDNA products from each exposure group indicating the abundance of rMuc-5AC and cyclophilin mRNA. Maxilloturbinates of control animals which normally have few mucous cells had detectable, but low levels of rMuc-5AC mRNA. Exposure to ozone resulted in marked increases of rMuc-5AC mRNA in these nasal tissues (Figure 2-11). There were 32% (1 day), 140% (2 day) or 126% (3 day) more rMuc-5AC mRNA in ozone-exposed rats, compared to air-exposed controls. The elevation of rMuc-5AC mRNA persisted in rats killed 2 days following the end of the 3-day-ozone exposure (70% more, compared to controls).

Figure 2-3. Light photomicrographs of maxilloturbinates from rats killed after 7-day exposure to 0-ppm ozone (filtered air) (A), or from rats killed 2 h after 1-day exposure (B), 2 h after 3-day exposure (C), or 4 days after 3-day exposure (D) to 0.5-ppm ozone. Tissues were stained with H&E. n = infiltrated neutrophil; arrowheads = basal lamina between epithelium and lamina propria; arrows = mucous cells; e = epithelium (NTE); tb = turbinate bone; v = blood vessel.

A
B
C
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fb
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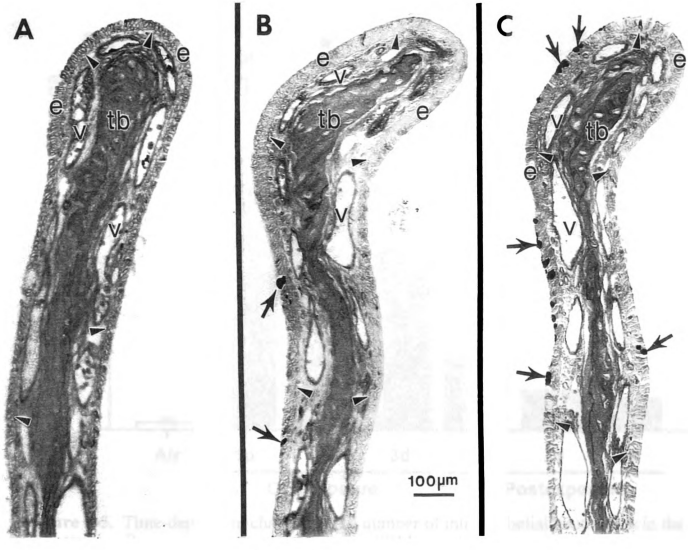
50 µm

Figure 2-4. Light photomicrographs of maxilloturbinates from rats killed after 7-day exposure to 0-ppm ozone (filtered air) (A), or from rats killed 2 h after 3-day exposure (B), or 4 days after 3-day exposure (C) to 0.5-ppm ozone. Tissues were stained with AB/PAS to detect acidic and neutral mucosubstances. Arrowheads = basal lamina between epithelium and lamina propria; arrows = AB/PAS-stained intrapeithlial mucosubstance in mucous cell; e = epithelium (NTE); tb = turbinate bone; v = blood vessel.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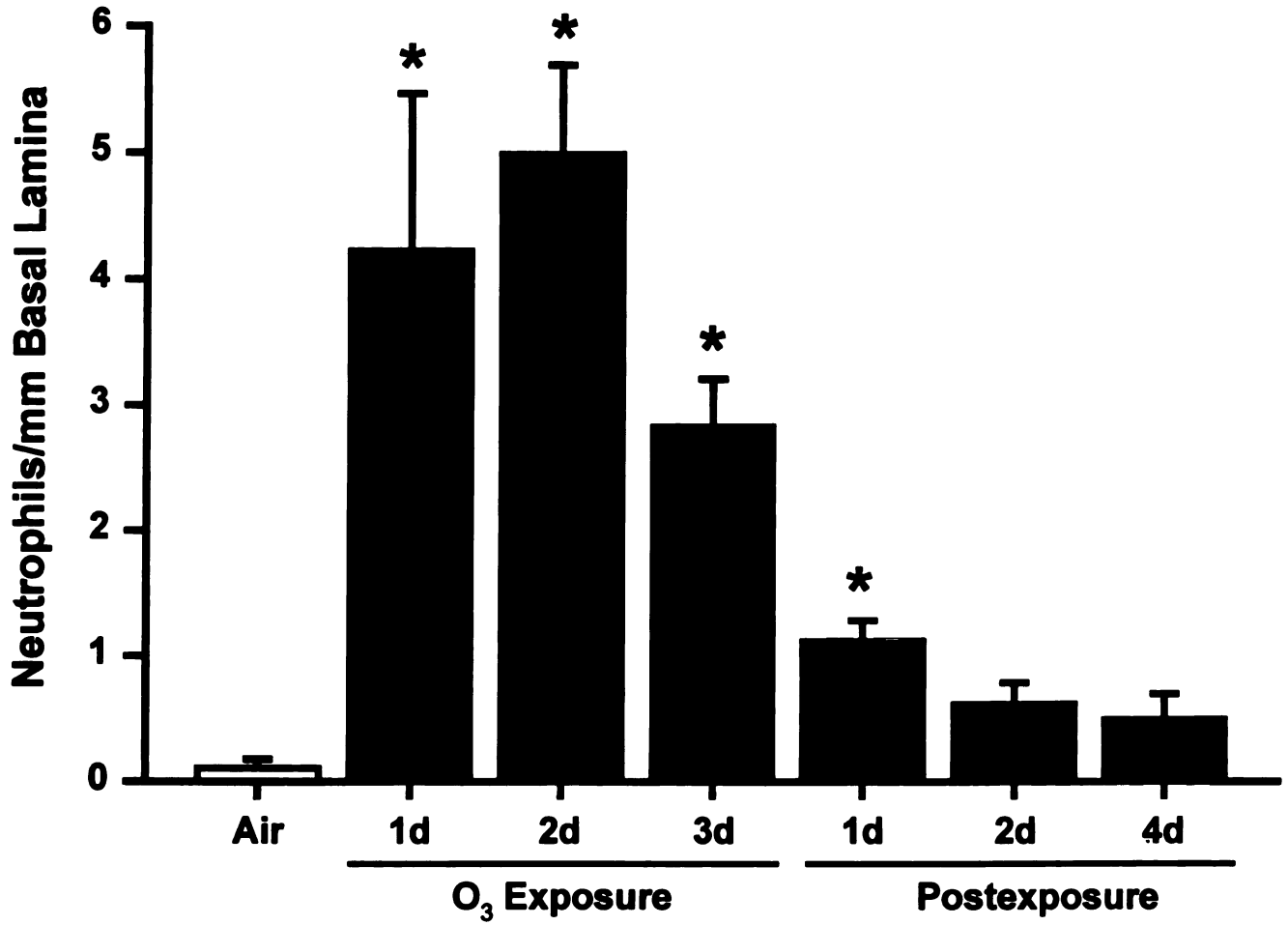

Figure 2-5. Time-dependent changes in the number of intraepithelial neutrophils in the NTE. Bars represent the group mean ± SEM (n = 8/group). *Significantly greater than air-exposed controls ($p \leq 0.05$).

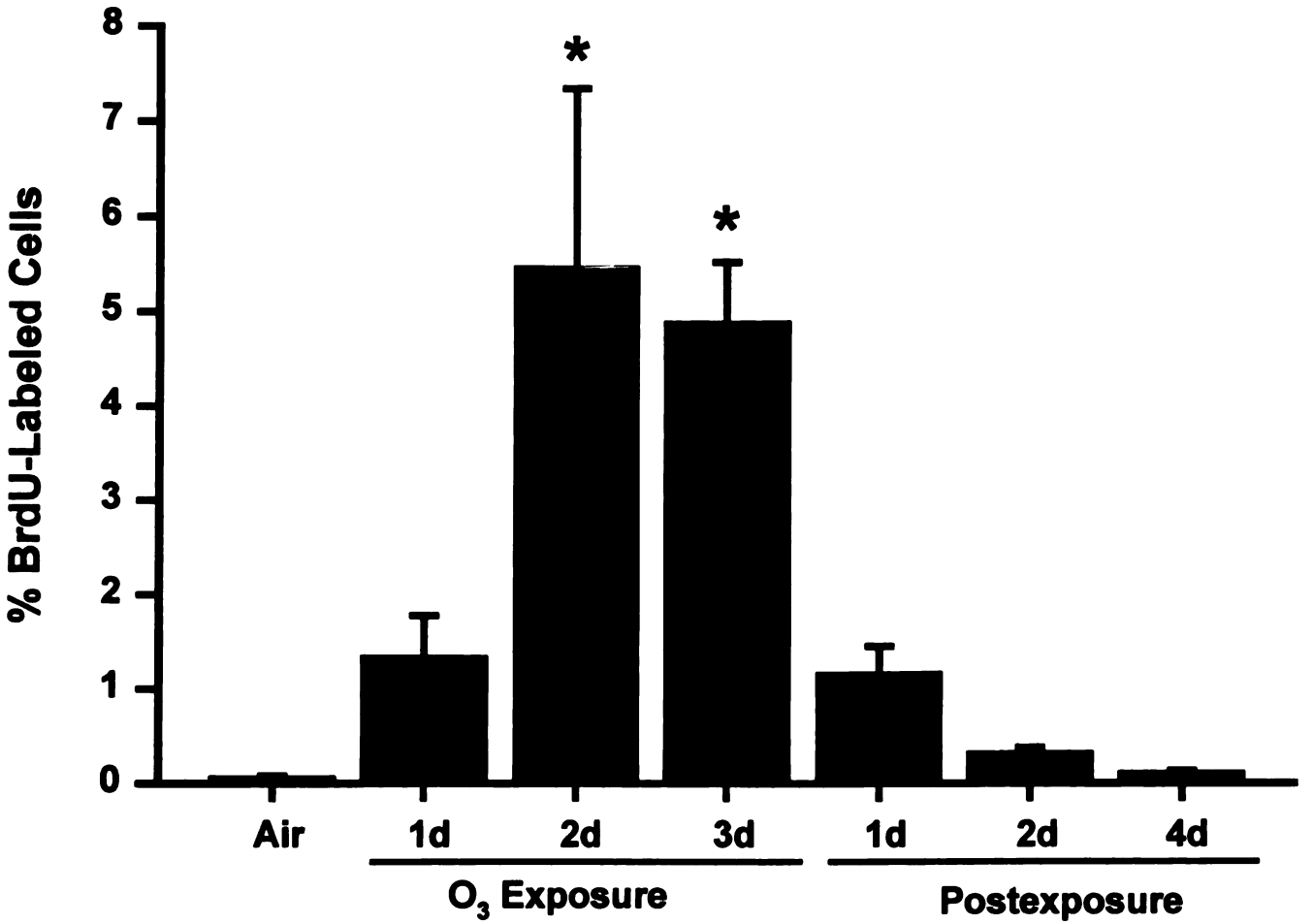

Figure 2-6. Time-dependent changes in the BrdU-labeling index (LI) in the NTE. Bars represent the group mean ± SEM (n = 8/group).*Significantly different from air-exposed controls ($p \leq 0.05$).

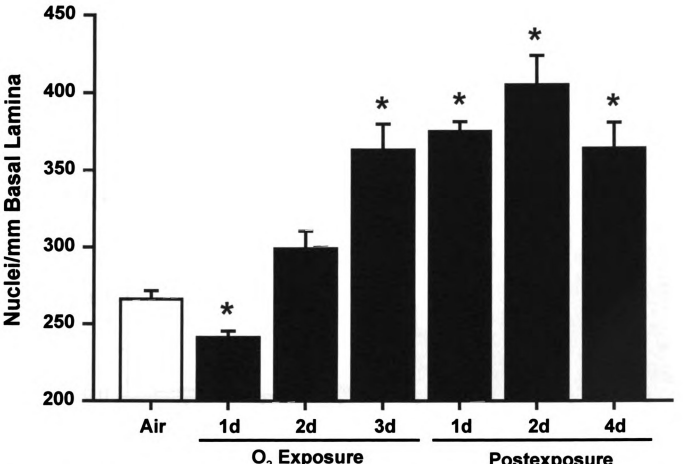

Figure 2-7. Time-dependent changes in the epithelialcell numeric density in the NTE. Bars represent the group mean $\pm$ SEM (n = 8/group).*Significantly different from air-exposed controls ($p \leq 0.05$).

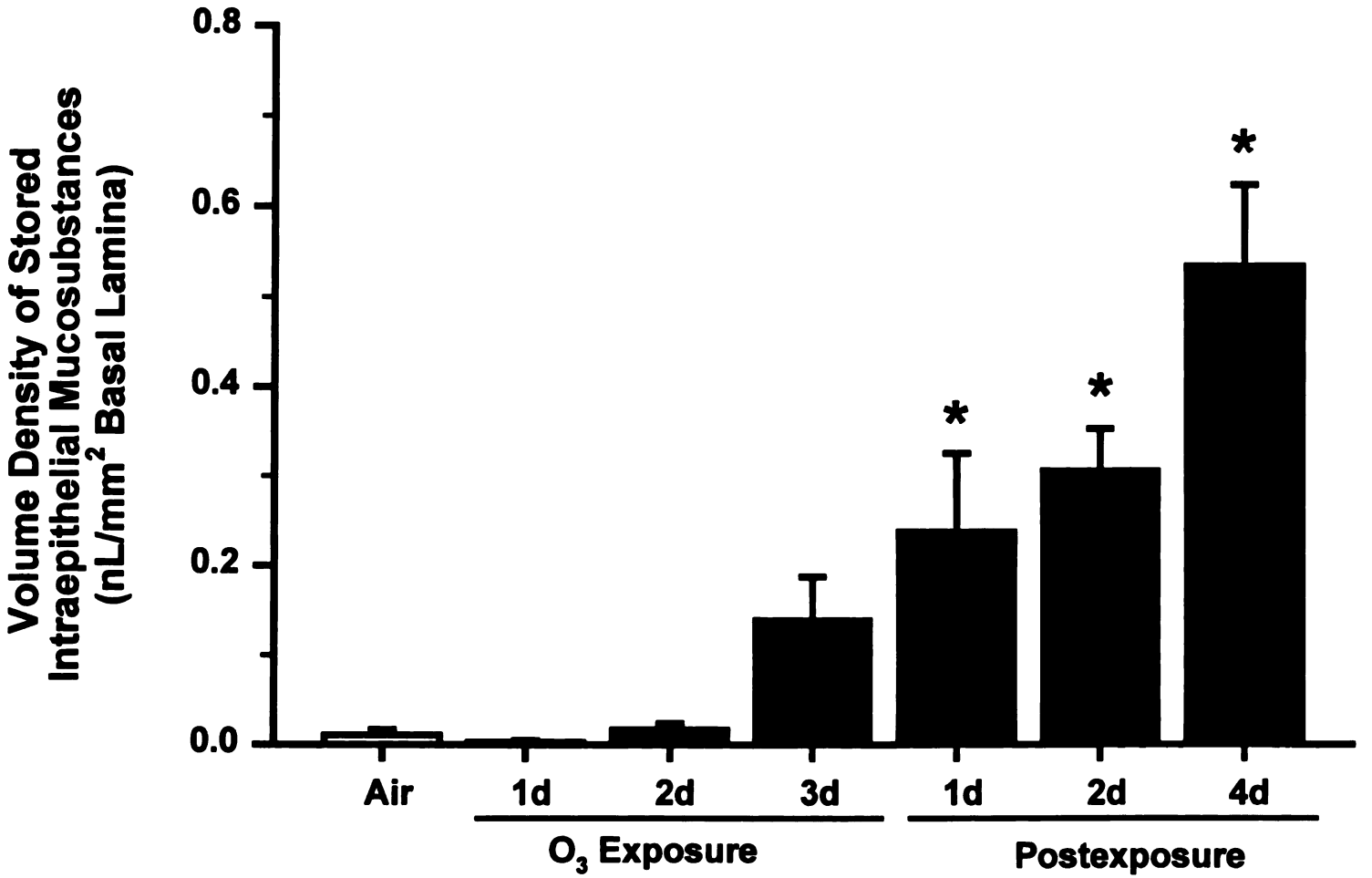

Figure 2-8. Time-dependent changes in the amount of stored mucosubstances in the NTE. Bars represent the group mean ± SEM (n = 8/group).*Significantly greater than air-exposed controls ($p \leq 0.05$).

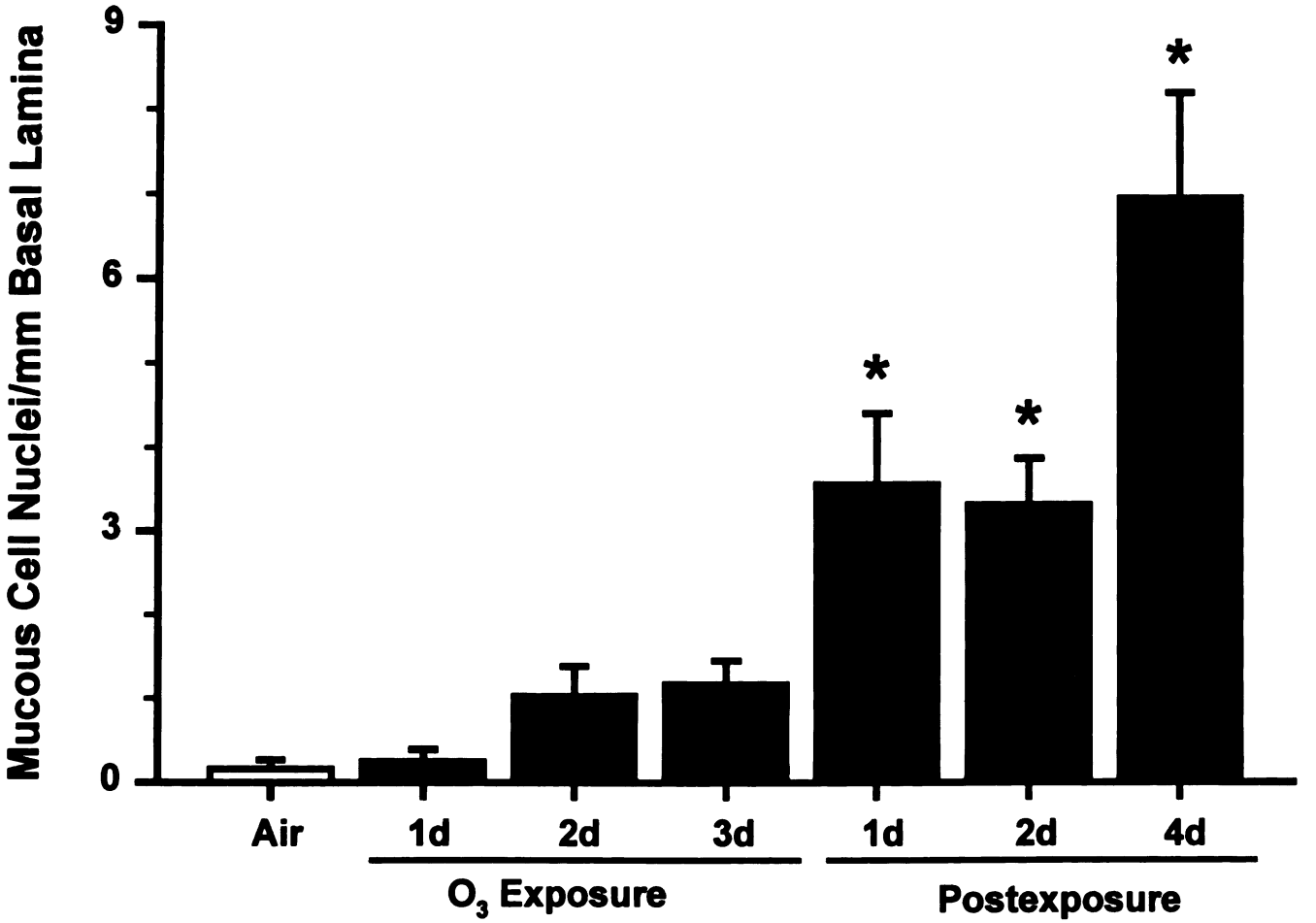

Figure 2-9. Time-dependent changes in the number of mucous cells in the NTE. Bars represent the group mean ± SEM (n = 8/group).*Significantly greater than air-exposed controls ($p \leq 0.05$).

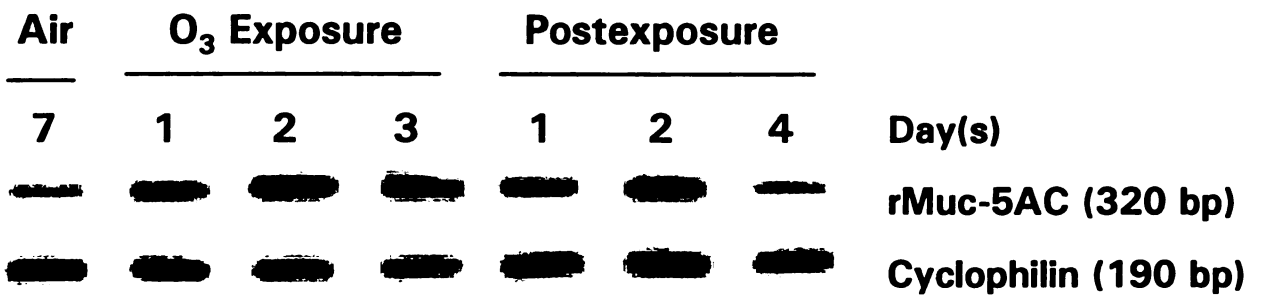

Figure 2-10. Digitized image of an ethidium bromide-stained agarose gel with representative RT-PCR cDNA bands for rMuc-5AC and cyclophilin from each exposure group (n = 8/group). cDNA was produced by reverse transcription of RNA extracted from one maxilloturbinate of each rat and PCR-amplification using primers specific for rat Muc-5AC and cyclophilin cDNA sequences.

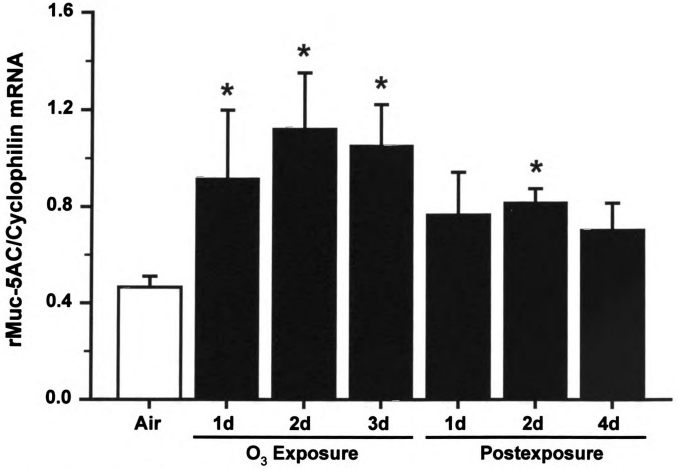

Figure 2-11. Time-dependent changes in the abundance of rMuc-5AC mRNA in maxilloturbinates. Bars represent the group mean $\pm$ SEM (n = 8/group).*Significantly greater than air-exposed controls ($p \leq 0.05$).

Discussion

The results of the present study demonstrate that a single 8-h exposure to 0.5 ppm ozone rapidly induces an increase in rMuc-5AC mRNA in the nasal mucosa of rats. This ozone-induced increase in rMuc-5AC mRNA preceded the morphologic appearance of increased number of mucous cells in the NTE by three days. In addition, the rMuc-5AC mRNA level remained elevated for two days after the end of 3 days of ozone exposure when MCM with copious amounts of intraepithelial mucosubstances was microscopically evident in the nasal epithelium. A conspicuous neutrophilic inflammatory response accompanied the ozone-induced increase in rMuc-5AC mRNA in the NTE after 1, 2, or 3 days of exposure. The ozone-induced neutrophilic inflammation was markedly attenuated 1 day after the end of the 3 days of exposure, and continued to decline during the postexposure period. In contrast, the severity of the ozone-induced mucous metaplastic changes increased during this same postexposure period. There was a time-dependent increase in the amount of intraepithelial mucosubstances and the number of mucous cells during the four days postexposure. This is the first report, to our knowledge, indicating that a single or repeated inhalation exposure to a high ambient concentration of ozone can induce an increase in the steady-state levels of an airway mucin-specific mRNA in the nasal airways of a laboratory animal. In addition, this study is the first to demonstrate that this ozone-induced increase in rMuc-5AC mRNA precedes, by several days, the onset of MCM in the affected nasal epithelium.

MCM in rat airway epithelium is also a prominent morphologic response to exposure to other airway irritants such as sulfur dioxide (Jany *et al.*, 1991), tobacco smoke (Lamb and Reid, 1969), bacterial endotoxin (Harkema and Hotchkiss, 1993; Shimizu *et al.*, 1996; Gordon *et al.*, 1996), 3-methylcholanthrene (Rehm and Kelloff, 1991), acrolein (Borchers and Leikauf, 1997), siloxane (Burns-Naas *et al.*, 1998), and chlorine (Wolf *et al.*, 1995). However, only a few previous studies have demonstrated irritant-induced alterations in mucin gene expression associated with MCM in airway epithelium of rats. Repeated exposure to sulfur dioxide induced early upregulation of mucin mRNA that persisted throughout the development of MCM in rat tracheobronchial epithelium (Jany *et al.*, 1991). Elevated rMuc-2 mRNA expression concurrent with MCM was also reported in tracheobronchial and pulmonary airway epithelium of rats after repeated exposure to acrolein (Borchers and Leikauf, 1997). These previous studies have reported elevated airway mucin mRNA levels in rat airways after repeated long-term (*i.e.*, >1 wk) inhalation exposure. The results of the present study suggest that ozone-induced alteration in mucin mRNA abundance is an early molecular predictor of mucous metaplastic changes, and probably plays a crucial role in the development of the phenotypic expression of mucous (goblet) cells in the nasal epithelium (*i.e.*, MCM).

Our present observations are supported by studies conducted i*n vitro* which examined mucous differentiation of airway epithelial cells (RTE) induced by retinoic acid, a major regulator of mucous differentiation, (Guzman *et al.*, 1996) or colonic epithelial cells (HT-29) induced by methotrexate, an inhibitor of nucleic acid metabolism (Lesuffleur *et al.*, 1993, 1990). The results from these studies have demonstrated that mucin messages were strongly expressed only in cultures that had undergone mucous cell differentiation. In

addition, there was a time-lag (2 - 7 days) between the first detection of mucin gene expression and that of mucus production during the *in vitro* mucous cell differentiation. Little is known about the kinetics of mucous biosynthesis in either normal or metaplastic mucous cells in airway epithelium. However, it is assumed that during the ozone-induced mucous metaplastic differentiation in the nasal epithelium, the mucin gene is activated in premetaplastic cells, and it takes time for (1) the synthesis of mucin core protein from the abundant mucin mRNA, (2) its glycosylation, and (3) the storage of the glycosylated mucin molecules into secretory granules of fully differentiated mucous cells. Further studies designed to determine the kinetics of rMuc-5AC mRNA accumulation, apomucin (protein core) synthesis and glycosylation are needed. Results from such studies will further our understanding of the underlying molecular mechanisms of ozone-induced MCM.

In the present study, we determined the time-dependent relationships of pre-metaplastic inflammatory and epithelial events (*i.e.*, neutrophilic inflammation, epithelial injury, regeneration and proliferation) in the nasal epithelium induced by single and repeated exposures to ozone. We have described how these temporal changes in the NTE correlate with the changes in mucin gene expression and the development of MCM. The coincidence of the onset of increased steady-state rMuc-5AC mRNA levels and the transient neutrophilic influx into the NTE, prior to the development of MCM, suggests that the early neutrophilic inflammatory response may be involved in the upregulation of mucin mRNA levels in the NTE and the initiation of MCM. Neutrophils, as well as airway epithelial cells, are significant sources of soluble mediators that can initiate or amplify inflammatory responses in airway tissues. We hypothesize that neutrophils play

an essential role in the ozone-induced mucin gene upregulation and ultimately in the pathogenesis of the MCM by releasing distinctive soluble mediators or by stimulating other resident cells (e.g., epithelial cells) to release inflammatory mediators. It has been known that soluble inflammatory mediators can rapidly modulate various cellular genes (e.g., genes for secondary mediators like cytokines) during airway injury and repair induced by inhaled toxicants, including ozone (Leikauf *et al.*, 1995; Levine, 1995; Nakamura *et al.*, 1992; Warner *et al.*, 1987; Marini *et al.*, 1992). Recently, several studies have focused on the investigation of the role of inflammatory mediators in the expression of airway mucin-specific genes. Cytokines such as TNF-α (Levine *et al.*, 1995), IL-6 (Levine *et al.*, 1994) and IL-4 (Temann *et al.*, 1997; Rankin *et al.*, 1996), or neutrophil proteases, specifically elastases (Voynow *et al.*, 1997), have been reported to induce mucin mRNA upregulation in airway epithelial cells *in vivo* or *in vitro*. Neutrophil elastase is a well-known mucous secretagogue and induces MCM in the airways of laboratory animals (Breuer *et al.*, 1985, 1993; Sommerhoff *et al.*, 1990; Jamil *et al.*, 1997; Kim *et al.*, 1987). Both soluble TNF-α and IL-6 induce mucin hypersecretion in airway epithelial cells *in vitro* at concentrations that also cause mucin gene upregulation (Levine *et al.*, 1994, 1995). Transgenic mice that overexpress IL-4 or IL-5 have MCM (Rankin *et al.*, 1996; Jain-Vora *et al.*, 1997; Lee *et al.*, 1997) and mucin hypersecretion (McBride *et al.*, 1994) with mucin gene upregulation in tracheobronchial or pulmonary airways. However, the precise roles of these inflammatory mediators in mucin gene expression or mucous cell function are still not clarified. Furthermore, few

studies have investigated the dependency of mediator-induced mucous differentiation or mucous overproduction on the activation of mucin genes.

Recently we demonstrated that an anti-inflammatory steroid, fluticasone propionate, decreased neutrophilic inflammation and MCM in the nasal epithelium of rats exposed to ozone (Hotchkiss *et al.*, 1998). Similarly, another steroid, dexamethasone has been shown to attenuate rat tracheal MCM induced by neutrophil lysates or elastase (Lundgren *et al.*, 1988). In addition, Kai *et al.* (1996) reported that dexamethasone suppressed the mucin mRNA expression and stored mucous product in airway epithelial cells in culture. These studies suggest a putative role of inflammatory cells in the induction of MCM, the upregulation of airway mucin genes, and the overproduction of mucins in airway epithelium. At present, steroids are among the most efficacious treatments for asthma (Barnes and Pedersen, 1993). However, only a few studies have documented the benefits of steroid therapy in alleviating the excessive production of airway mucus (Marom *et al.*, 1984; Shimura *et al.*, 1990; Lundgren *et al.*, 1988). In addition, it is not certain whether or not steroids modulate the mucin gene expression directly by acting on glucocorticoid regulatory elements present in the mucin gene or indirectly through other anti-inflammatory mechanisms in airway tissues.

The present study was also designed to examine the kinetics of epithelial injury, regeneration and proliferative adaptation (*i.e.*, hyperplasia), and especially the time-dependent relationships of these ozone-induced epithelial changes with mucin gene expression and MCM. Interestingly, the severity and temporal pattern of nasal epithelial cell loss, subsequent burst of DNA synthesis, and cell proliferation leading to epithelial repair during single and repeated daily exposure to ozone were similar to those

determined after a single exposure in a previous study reported by Hotchkiss *et al.* (1997). In that study, rats were exposed to 0.5 ppm ozone once for 8 h, and the epithelial responses were examined 2 - 36 h postexposure. Even though rats in our study received repeated ozone exposures, temporal relationship of epithelial DNA synthesis and the proliferation of injured NTE cells observed after 2 and 3 days of exposure were similar to those observed in the previous study at 24 and 36 h after the single exposure to ozone. The results from both studies indicate that the induced cellular renewal mechanisms in the NTE following the first day of ozone exposure are not significantly affected by subsequent ozone exposure. The epithelial regeneration after 2 days of exposure and subsequent hyperproliferative response (*i.e.*, epithelial hyperplasia) were concurrent with increased levels of rMuc-5AC mRNA in the NTE. It is plausible that new epithelial cells with abundant mucin message repopulate the injured epithelium and are responsible for the observed increase in mucin mRNA in the regenerative and hyperplastic epithelium. It is also possible that NTE cells which survive the initial ozone exposure are stimulated by ozone to upregulate their normally low-constitutive levels of mucin mRNA. The exact cellular mechanisms responsible for ozone-induced upregulation of rMuc-5AC mRNA in the NTE cannot be determined from the results of our present study. Further studies, using *in situ* hybridization and immunohistochemistry techniques, are needed to identify the NTE cells that express rMuc-5AC mRNA and produce mucin protein during regeneration and hyperproliferation after ozone exposure.

Because the onset of epithelial proliferation preceded the appearance of increased numbers of mucous (goblet) cells, it is possible that epithelial cell proliferation is a prerequisite for MCM. However, the dependency of MCM on the preceding epithelial

proliferative responses cannot be determined from our present results. In our study, neutrophilic inflammation preceded both the hyperplastic and metaplastic responses in the ozone-exposed NTE. Though the role of neutrophils in the repair and hyperproliferation of NTE is unknown, other studies in the literature suggest that these inflammatory cells are important in airway epithelial repair following ozone-induced injury in the lungs of laboratory animals (Pino *et al.*, 1992; Hyde *et al.*, 1992).

In conclusion, acute ozone exposure induced increased levels of rMuc-5AC mRNA in the NTE within hours after the start of exposure. This ozone-induced upregulation of the airway mucin gene was observed several days before the phenotypic expression and the intraepithelial production and storage of mucosubstances (*i.e.*, MCM). Mucin gene upregulation occurred concurrently with ozone-induced neutrophilic inflammation in the NTE but was maintained even after the initial neutrophilic inflammation was resolved 2 days later. Although temporal correlations of epithelial and inflammatory responses in the present study do not prove causality, our results indicate that (1) upregulation of mucin mRNA by acute ozone exposure is associated with the concurrent neutrophilic inflammation and epithelial hyperplasia in the NTE, and that (2) ozone-induced MCM may be dependent on these important pre-metaplastic responses (*i.e.*, mucin mRNA upregulation, neutrophilic inflammation and epithelial proliferation).

CHAPTER 3

Neutrophil-Dependent and -Independent Alterations in the Nasal Epithelium of Ozone-Exposed Rats.

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Abstract

Ozone induces epithelial hyperplasia and mucous cell metaplasia (MCM) in nasal transitional epithelium (NTE) of rats. A transient neutrophilic influx accompanies upregulation of mucin mRNA prior to the onset of MCM. The present study was designed to examine the role of neutrophils in ozone-induced epithelial changes in the NTE of rats. Fourteen h prior to inhalation exposure, male F344/N rats were treated *ip* with anti-rat neutrophil antiserum or control serum. For morphometric analyses, antiserum- or control serum-treated rats were exposed to 0 (filtered air) or 0.5 ppm ozone for 3 days (8 h/day). At the end of exposure, rats were treated *ip* with 5'-bromo-2-deoxyuridine (BrdU) to label epithelial cells undergoing DNA synthesis, and killed 2 h or 4 days later. Nasal tissues were processed to morphometrically determine the BrdU-labeling index, the numeric densities of neutrophils, total epithelial cells and mucous cells, and the amount of intraepithelial mucosubstances (IM) in the NTE. For rMuc-5AC mRNA analysis, antiserum- or control serum-treated rats were exposure to 0 or 0.5 ppm ozone for 1 or 3 days (8 h/day). Rats were killed immediately after 1 or 3 days of exposure, or 4 days after 3 days of exposure, and RNA was isolated from microdissected maxilloturbinates. At 2 h after 3-day exposure, rats treated with antiserum had ~ 90% fewer circulating neutrophils than rats treated with control serum. Antiserum-treated/ozone-exposed rats had 87% less infiltrating neutrophils than control serum-treated/ozone-exposed rats. At 4 days after 3-day exposure, antiserum-treated/ozone-exposed rats had 66% less IM and 58% fewer mucous cells in the NTE than did control serum-treated/ozone-exposed rats. Antiserum treatment had no effects on ozone-induced

epithelial cell proliferation and mucin mRNA upregulation. The results of this study indicated that ozone-induced MCM was neutrophil-dependent, while ozone-induced epithelial cell proliferation and mucin gene upregulation were neutrophil-independent.

Introduction

Both cellular inflammation and overproduction/hypersecretion of airway mucus are thought to be important factors in the pathogenesis of many obstructive pulmonary disorders, including acute and chronic bronchitis (Reid, 1954; Robbins *et al.*, 1984), asthma (Aikawa *et al.*, 1992), cystic fibrosis (Chartrard and Marks, 1994), and upper respiratory tract disorders such as allergic rhinitis (Robbins *et al.*, 1984). Similar changes have also been induced in airway mucosa of laboratory animals by inhaled irritants such as sulfur dioxide, cigarette smoke, and bacterial endotoxin (Harkema and Hotchkiss, 1993; Lamb and Reid, 1969; Jany *et al.*, 1991).

Ozone, the major oxidant air pollutant in photochemical smog, causes inflammation and tissue damage in human airways, including the nose (Calderon Garciduenas *et al.*, 1992; Koren *et al.*, 1990; Peden *et al.*, 1995). In F344/N rats, we have demonstrated that short-term (*i.e.*, days) (Hotchkiss *et al.*, 1989; Harkema *et al.*, 1989) or long-term (*i.e.*, weeks or months) (Harkema *et al.*, 1994, 1997) exposures to high ambient concentrations of ozone (0.5 - 1.0 ppm) induce marked mucous cell metaplasia (MCM) with accompanying increases in stored intraepithelial mucosubstances and number of

epithelial cells (*i.e.*, epithelial hyperplasia) in nasal airways. These ozone-induced epithelial alterations were restricted to the nasal transitional epithelium (NTE), which is normally devoid of mucous cells, lining the lateral meatus of the proximal aspect of the nasal cavity (Harkema *et al.*, 1989, 1992; Hotchkiss *et al.*, 1991). A marked and transient neutrophilic influx into the NTE preceded the epithelial hyperplasia and MCM induced by repeated ozone exposure (Harkema *et al.*, 1989; Hotchkiss *et al.*, 1997). More recently, we have also demonstrated that acute ozone exposure results in an increase in the steady-state level of an airway mucin-specific (rMuc-5AC) mRNA prior to the onset of the MCM in rat NTE (Cho *et al.*, 1997). In addition, the mucin gene upregulation (a putative molecular predictor of MCM) as well as the burst of epithelial DNA synthesis (a marker of epithelial cell injury and proliferation) coincide with the neutrophilic inflammation in the NTE (Hotchkiss *et al.*, 1997; Cho *et al.*, 1997).

Neutrophils have been implicated as a cause of tissue damage in a number of airway inflammatory diseases. Activation of neutrophils results in the release of powerful inflammatory mediators that may damage both cellular and extracellular tissue components and amplify the inflammatory response (Weiss, 1989; Okrent *et al.*, 1990; Sibille and Reynolds, 1990). The involvement of neutrophils in ozone-induced injury and repair has been investigated in distal airways of several laboratory animal species (Pino *et al.*, 1992; Hyde *et al.*, 1992; Salmon *et al.*, 1998). However, the role of neutrophils in the pathogenesis of ozone-induced nasal airway lesions in rats has not been investigated.

The present study was designed to test the hypothesis that neutrophilic inflammation plays an important role in ozone-induced epithelial alterations (*i.e.*, hyperplasia and

MCM) as well as mucin gene upregulation in rat nasal airways. For this purpose, we depleted rats of their circulating neutrophils using an anti-rat neutrophil antiserum prior to repeated, acute, ozone exposure. By removing the circulating pool of neutrophils, we were able to examine the contribution of these inflammatory cells to the pathogenesis of ozone-induced proliferative and metaplastic alterations in the NTE. The results of this study provided new insights into the underlying mechanisms of ozone-induced injury, adaptation, and repair of airway epithelium after short-term exposure to ozone.

Materials and Methods

Animals, neutrophil depletion, and exposure. One hundred and twenty male F344/N rats (Harlan Sprague-Dawley, Indianapolis, IN), 10 - 12 week old, were used in this study. To morphometrically determine the effect of neutrophil-depletion on ozone-induced epithelial proliferation and MCM, 48 rats were randomly assigned into one of 8 experimental groups (n = 6/group) (Table 3-1A). To determine the effect of neutrophil-depletion on ozone-induced increases in the steady-state levels of rMuc-5AC mRNA, 72 rats were randomly divided into one of 12 experimental groups (n = 6/group) (Table 3-1B). Rats were housed two per cage in polycarbonate shoebox-type cages with Cell-Sorb Plus bedding (A&W Products, Inc., Cincinnati, OH) and filter caps. Water and food (Tek Lab 1640; Harlan Sprague Dawley, Indianapolis, IN) were available *ad libitum*. Rats

Table 3-1. Experimental groups and animal assignment.

A. Morphometric Analyses

Postexposure Time	0 ppm (Filtered Air)		0.5 ppm	
	Control Serum	Antiserum	Control Serum	Antiserum
2 h after 3-day exposure	6*	6	6	6
4 days after 3-day exposure	6	6	6	6

B. Mucin (rMuc-5AC) mRNA Analysis

Postexposure Time	0 ppm (Filtered Air)		0.5 ppm	
	Control Serum	Antiserum	Control Serum	Antiserum
2 h after 1-day exposure	6	6	6	6
2 h after 3-day exposure	6	6	6	6
4 days after 3-day exposure	6	6	6	6

* Animal number.

were maintained on a 12-h light/dark cycle beginning at 6:00 a.m. under controlled temperature (16 - 25°C) and humidity (40 - 70%). Rats were conditioned in whole-body exposure chambers (HC-1000, Lab Products, Maywood, NJ) supplied with filtered air for 1 day prior to the start of the inhalation exposure as described in Chapter 2.

Fourteen h prior to inhalation exposure, the rats were briefly anesthetized with 4% halothane in oxygen, and half of the rats were depleted of circulating neutrophils using an intraperitoneal (*ip*) injection of 1 ml rabbit anti-rat neutrophil antiserum (antiserum; Accurate Scientific Corp., Westbury, NY). In normal rats, a single *ip* injection of this antiserum is known to deplete the number of circulating blood neutrophils to below 1% of normal levels in 12 h, and the depletion persists for up to 5 days post-injection (Snipes *et al.*, 1995). The antiserum is specific for mature neutrophils without damaging cellular precursors in the bone marrow or other blood components such as red blood cells (Snipes *et al.*, 1995; Davis *et al.*, 1969). The remaining rats were treated *ip* with 1 ml of normal rabbit serum (control serum; Accurate Scientific Corp., Westbury, NY).

The control serum- or antiserum-treated rats designated for morphometric analyses were exposed to either 0.5 ppm ozone or filtered air (0 ppm), 8 h/day, for 3 days. The other rats designated for mucin-specific mRNA analysis were exposed to either 0.5 ppm ozone or filtered air (0 ppm) for 1, 3, or 7 days. All the rats were exposed daily to ozone or filtered air in the whole-body exposure chambers from 6 am to 2 pm. Ozone was generated with an OREC Model O3VI-O ozonizer (Ozone Research and Equipment Corp., Phoenix, AZ) as explained in detail in Chapter 2. The chamber ozone concentrations (mean $\pm$ standard deviation) for 1 - 3 days of exposure to 0.5 ppm-ozone

were 0.500 ± 0.008. The chamber ozone concentrations during exposures to filtered air were maintained less than 0.05 ppm.

Blood collection and assessment of circulating neutrophils. At the end of 3 days of exposure, all the rats exposed for the morphometric analyses were treated *ip* with 5'-bromo-2-deoxyuridine (BrdU; 50 mg/Kg body wt.) to label cells undergoing DNA synthesis in the S-phase of the cell cycle. At 2 h or 4 days later, rats were anesthetized by 4% halothane in oxygen, and approximately 2-ml of blood were drawn from the *vena cava* or the left ventricle of the heart of each rat to assess the number of circulating neutrophils. Blood was collected in evacuated blood collection tubes (Becton Dickenson, Rutherford, NJ) containing K3-ethylenediaminetetraacetic acid (EDTA). The number of nucleated cells per cubic millimeter of blood was measured with a Serono-Baker System 9000 automated cell counter (Serono-Baker Diagnostics, Allentown, PA). Differential counts of leukocytes were determined by counting 100 nucleated white blood cells from blood smears stained with Wright-Giemsa stain (Diff-Quik; Baxter, McGaw Park, IL). The total number of neutrophils per cubic millimeter of blood was determined by multiplying the percent occurrence of neutrophils (*i.e.*, the number of neutrophils per 100 white blood cells) by the total number of nucleated white blood cells per cubic millimeter of blood. After collecting the blood, rats were killed by exsanguination via the abdominal aorta.

Necropsy and tissue preparation for morphometric analyses. After death, the head of each rat was removed from the carcass, and the nasal airways were flushed retrograde through the nasopharyngeal orifice with 5 ml of zinc formalin (Anatech, Ltd., Kalamazoo, MI). After the eyes, lower jaw, skin and musculature were removed from the head, the nasal tissues were stored in a large volume of the same fixative for a minimum of 48 h.

The zinc formalin-fixed nasal tissues were decalcified in 13% formic acid for 4 days, and then rinsed in tap water for 2 h as previously described by Harkema *et al.* (1988). A tissue block was removed from the proximal aspect of the nasal cavity by making two transverse cuts perpendicular to the hard plate. The first cut was immediately posterior to the upper incisor tooth (Figure 2-1A in page 49), and the second cut was at the level of the incisive papilla. The tissue block was excised, embedded in paraffin, and 5 μm-thick sections were cut from the anterior face of the tissue block.

Nasal tissue sections from each tissue block were histochemically stained with hematoxylin and eosin for morphological identification of epithelial cells or Alcian Blue (pH 2.5)/Periodic Acid-Schiff's sequence (AB/PAS) to identify acidic and neutral mucosubstances in the surface epithelium, and immunohistochemically stained with anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) to detect BrdU-labeled nuclei (Johnson *et al.*, 1990).

Morphometry of neutrophilic inflammation, epithelial cell numeric density and DNA synthesis. The NTE overlying the maxilloturbinate of each animal was analyzed using computerized image analysis and standard morphometric techniques (Hotchkiss and Harkema, 1992; Hotchkiss *et al.*, 1991). Neutrophilic inflammation (intraepithelial neutrophils/mm basal lamina), epithelial cell labeling index (LI; % BrdU-labeled epithelial cell nuclei) and epithelial cell numeric density (epithelial nuclei/mm of basal lamina) were determined using a Power Macintosh 7100/66 computer and the public domain image analysis software (NIH Image; written by Wayne Rasband at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) as described in detail in Chapter 2.

Morphometry of stored intraepithelial mucosubstances and mucous cells. The volume density (Vs) of AB/PAS-stained mucosubstances and the numeric cell densities of mucous cells (epithelial cells containing AB/PAS-stained mucosubstances) in the NTE lining the maxilloturbinates were determined using computerized image analysis and standard morphometric techniques as explained in Chapter 2.

Necropsy and microdissection of tissues for mucin mRNA analysis. For mucin mRNA analysis, rats exposed to ozone or filtered air for 1 day were killed immediately after the end of exposure. Rats exposed to ozone or filtered air for 3 days were killed immediately or 4 days after the end of exposure. We chose these three time points for rMuc-5AC mRNA analysis based on our previous findings that ozone induces mucin mRNA upregulation after 1 day of ozone exposure (8 h, 0.5 ppm) and this increased mucin

mRNA level persists 2 days postexposure (Cho *et al.*, 1997). The head of each rat was removed and the nasal airways were opened by splitting the nose in a sagittal plane adjacent to the midline. Maxilloturbinates were excised by microdissection from both nasal passages of each head (Figure 2-1A in page 49) as described in Chapter 2, and immediately homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH) to isolate total RNA and analyze the abundance of mucin (rMuc-5AC) mRNA.

Analysis for mucin mRNA in nasal tissues. Total cellular RNA was isolated from the maxilloturbinate homogenate according to the method of Chomczynski *et al.* (1987) following the procedure in Chapter 2. The RNA was then analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) with rat MUC-5AC-specific primers to determine the steady-state levels of rMuc-5AC mRNA in maxilloturbinates following the procedures described in Chapter 2. Cyclophilin mRNA was used as an internal standard in this semi-quantitative RT-PCR analysis. The abundance of mucin mRNA was determined by densitometric analysis as described in Chapter 2.

Statistical analyses. All data were expressed as the mean group value ± the standard error of the mean (SEM). The data were log transformed to make the variances approximately equal for all groups. The data from morphometric analyses were analyzed by three-way analysis of variance (ANOVA) to determine the potential effects of exposure atmosphere (filtered air or ozone), type of serum injection (control serum or antiserum), and postexposure time (2 h or 4 days) on circulating neutrophils, neutrophilic

influx, epithelial proliferation, and MCM. Student-Newman-Keuls Method, an all pairwise multiple comparison procedure, was then used to determine the significant differences in group mean values. The data from rMuc-5AC mRNA analysis were first analyzed by three-way ANOVA as described above. Only the exposure atmosphere and the type of serum injection were identified as factors contributing to variances in group mean mRNA levels. Therefore, data from similarly exposed and injected experimental groups (e.g., antiserum-treated/air-exposed) that were sacrificed at different times were combined. The pooled data were then analyzed by Analysis of Contrasts to determine the differences in the mean rMuc-5AC mRNA levels among the combined experimental groups. Statistical analyses were performed using a commercial statistical analysis package (SigmaStat; Jandel Scientific Software, San Rafael, CA). The criterion for statistical significance was set at $p \leq 0.05$ at all analyses.

Results

Number of circulating blood neutrophils. The *ip* injection of anti-neutrophil antiserum dramatically decreased the numbers of circulating neutrophils to 12% or 6% of those in control serum-treated rats exposed to air or ozone, respectively, at 2 h after 3 days of exposure (*i.e.*, 4 days after antiserum treatment) (Figure 3-1). At 4 days postexposure (*i.e.*, 8 days after antiserum treatment), the number of circulating neutrophils in antiserum-treated/air-exposed rats was not significantly different from that

in air-exposed/control serum-treated rats. However, there was a slight increase (18%) in the number of circulating neutrophils in antiserum-treated/ozone-exposed rats killed at 4 days postexposure, compared to control serum-treated/ozone-exposed rats. There were no significant differences in the numbers of circulating neutrophils in air- or ozone-exposed rats treated with control serum at either postexposure time.

Nasal histopathology. No exposure-related lesions were observed microscopically in the nasal mucosa of rats treated with either control serum or antiserum and exposed to filtered air (0 ppm ozone). The nasal lesions in rats exposed to 0.5 ppm ozone were restricted to the mucosa containing the NTE that lined the lateral meatus in the proximal nasal cavity of both antiserum- and control serum-treated rats.

Two h after 3 consecutive days of exposure to 0.5 ppm ozone, the principal feature in the NTE of control serum-treated rats was the appearance of widely scattered mitotic figures and epithelial hyperplasia. These rats had NTE that was approximately 3 - 4 cells in thickness. In contrast, control serum-treated/air-exposed rats had NTE that was 1 - 2 cells in thickness. Concurrent with the ozone-induced epithelial hyperplasia was a mild inflammatory response in the nasal mucosa that was characterized by endothelial margination of neutrophils in the large capacitance vessels of the lamina propria and an influx of neutrophils in both the lamina propria and NTE. These ozone-induced alterations in the NTE were most noticeable in the dorsal and medial aspects of the maxilloturbinate, the lateral ridge of the nasoturbinate and the dorsal recess of the lateral wall. Rats in the antiserum-treated/ozone-exposed group also had conspicuous mitotic figures and epithelial hyperplasia (3 - 4 cells in thickness) in the NTE like those in the

control serum-treated/ozone-exposed rats. However, there was no associated inflammatory cell influx in the hyperplastic NTE of the antiserum-treated/ozone-exposed rats.

At 4 days after 3 days of ozone exposure, the principal feature in the rats treated with control serum was MCM characterized by copious AB/PAS-stained mucosubstances in the mucous cells in the NTE. The ozone-induced MCM in the NTE was most severe in the dorsal and medial aspects of the maxilloturbinates. Hyperplasia was still evident in the NTE of these rats. In contrast, antiserum-treated/ozone-exposed rats had only a few scattered AB/PAS-stained mucous cells in the hyperplastic NTE. One or two rat(s) in this exposure group had no AB/PAS-positive mucous cells in the NTE lining one maxilloturbinate. No intraepithelial neutrophils were evident in the NTE of all the rats killed 4 days postexposure. Figures 3-2 and 3-3 illustrate morphologic similarities and differences in the NTE lining the maxilloturbinates from air-control, ozone-exposed/control serum-treated and ozone-exposed/antiserum-treated rats killed at 4 days postexposure.

Neutrophilic inflammation. Figure 3-4 illustrates the number of intraepithelial neutrophils in the NTE 2 h or 4 days after the end of 3 days of exposure to filtered air or 0.5 ppm ozone. At 2 h postexposure, control serum-treated/ozone-exposed rats had significantly more intraepithelial neutrophils in the NTE, compared to control serum-treated/air-exposed rats (16-times greater). At the same time point, antiserum-treated/ozone-exposed rats had markedly fewer intraepithelial neutrophils (87% less), compared to control serum-treated/ozone-exposed rats. The numeric density of

intraepithelial neutrophils was, however, still significantly greater (3.5-times) than that of antiserum-treated/air-exposed rats. Four days following the last exposure, there was no difference in the numbers of intraepithelial neutrophils in all rats, independent of inhalation exposure and type of serum treated. All air-exposed rats had few neutrophils in the NTE at both postexposure times.

Epithelial cell proliferation. Ozone induced significant increases in the NTE cell labeling index in both control serum-treated and antiserum-treated rats (20- and 2.8-times greater than that in corresponding air-exposed controls, respectively) sacrificed 2 h after the end of exposure (Figure 3-5). There was, however, no significant difference between the NTE cell labeling index of ozone-exposed rats treated with control serum and antiserum at this time point. Four days after the end of exposure, the NTE cell labeling index of the ozone-exposed rats was not significantly different from that of air-exposed control rats (0 - 2 labeled cells/animal), regardless of the type of serum injected. Air-exposed/antiserum-treated rats sacrificed 2 h after the end of exposure had significantly increased NTE labeling index (6-times) than air-exposed/control serum-treated controls.

Two h after the end of exposure, ozone induced a significant increase in the number of NTE cells in rats treated with control serum (31%), but not in antiserum-treated rats, compared to air-exposed rats treated with same type of serum (Figure 3-6). At 4 days postexposure, ozone-exposed rats treated with either control serum or antiserum had significantly more NTE cells (35% and 31%, respectively), compared to air-exposed rats treated with same type of serum. There was no significant difference in the number of

NTE cells between air-exposed rats sacrificed at either time point, regardless of the type of serum injected.

Mucous cell metaplasia. At 4 days postexposure, ozone-exposed/control serum-treated rats had significantly more intraepithelially stored mucosubstances (100-fold) and mucous cells (18-fold) in the NTE than did air-exposed/control serum-treated rats (Figures 3-7 and 3-8). Ozone-exposed/antiserum-treated rats had significantly less intraepithelial mucosubstances and fewer mucous cells in the NTE (66% and 58%, respectively) than ozone-exposed/control serum-treated rats sacrificed at the same postexposure time. However, these ozone-exposed/antiserum-treated rats still had significantly more intraepithelial mucosubstances (35-fold) and mucous cells (7-fold) than air-exposed/antiserum-treated rats. Air-exposed rats injected with either control serum or antiserum had few mucous cells and little intraepithelial mucosubstances in the NTE. At 2 h postexposure, little stored intraepithelial mucosubstances and few mucous cells were detected in any experimental group.

rMuc-5AC mRNA expression. Ozone exposure induced a significant increase in steady-state levels of rMuc-5AC mRNA in both control serum-treated (141%) and antiserum-treated (58%) rats, compared to air-exposed control rats treated with the same type of serum (Figure 3-9). Air-exposed rats treated with antiserum had 2-fold greater steady-state rMuc-5AC mRNA levels than air-exposed rats treated with control serum.

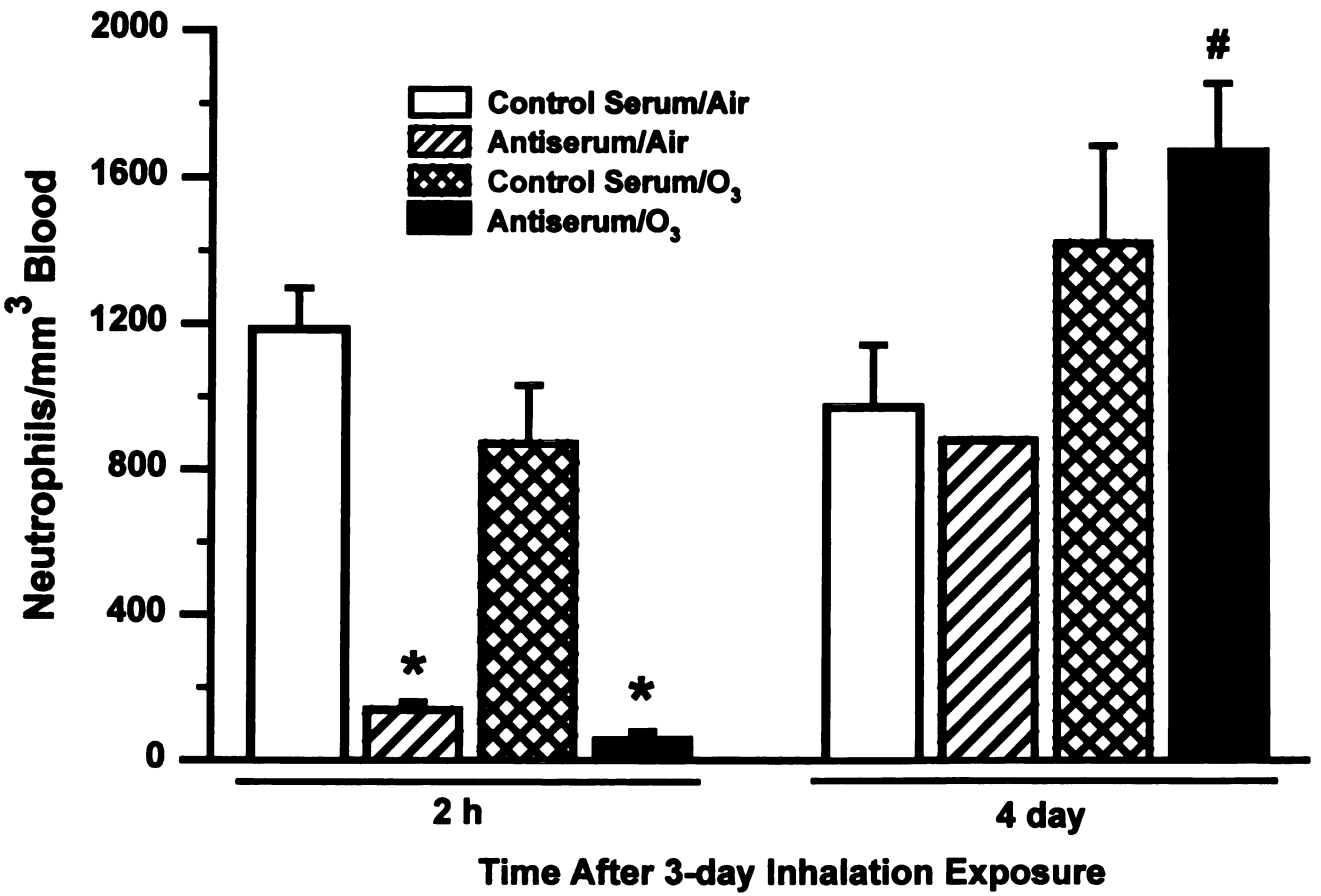

Figure 3-1. Effect of antiserum treatment on the number of circulating blood neutrophils. Bars represent the group mean ± SEM (n = 6/group). *Significantly different from control serum-treated/ similarly-exposed rats killed at the same time point after exposure ($p \leq 0.05$). #Significantly different from antiserum-treated/air-exposed rats killed at 4 days postexposure ($p \leq 0.05$).

Figure 3-2. Light photomicrographs of maxilloturbinates from rats treated with control serum and killed 4 days after 3-day exposure to 0-ppm ozone (filtered air) (A), or rats treated with control serum (B), or with antiserum (C) and killed 4 days after 3 days of exposure to 0.5-ppm ozone. Tissues were stained with H&E. Arrowheads = basal lamina between epithelium and lamina propria; arrows = mucous cells; e = epithelium (NTE); TB = turbinate bone; v = blood vessel.

A
B
C
e
V
TB
100µm

Figure 3-3. Light photomicrographs of maxilloturbinates from rats treated with control serum and killed 4 days after 3-day exposure to 0-ppm ozone (filtered air) (A), or rats treated with control serum (B) or with antiserum (C) and killed 4 days after 3 days of exposure to 0.5-ppm ozone. Tissues were stained with AB/PAS to detect acidic and neutral mucosubstances. Arrowheads = basal lamina between epithelium and lamina propria; arrows = AB/PAS-stained mucosubstances; e = epithelium (NTE); TB = turbinate bone; v = blood vessel.

A
B
C
e
v
TB
100µm

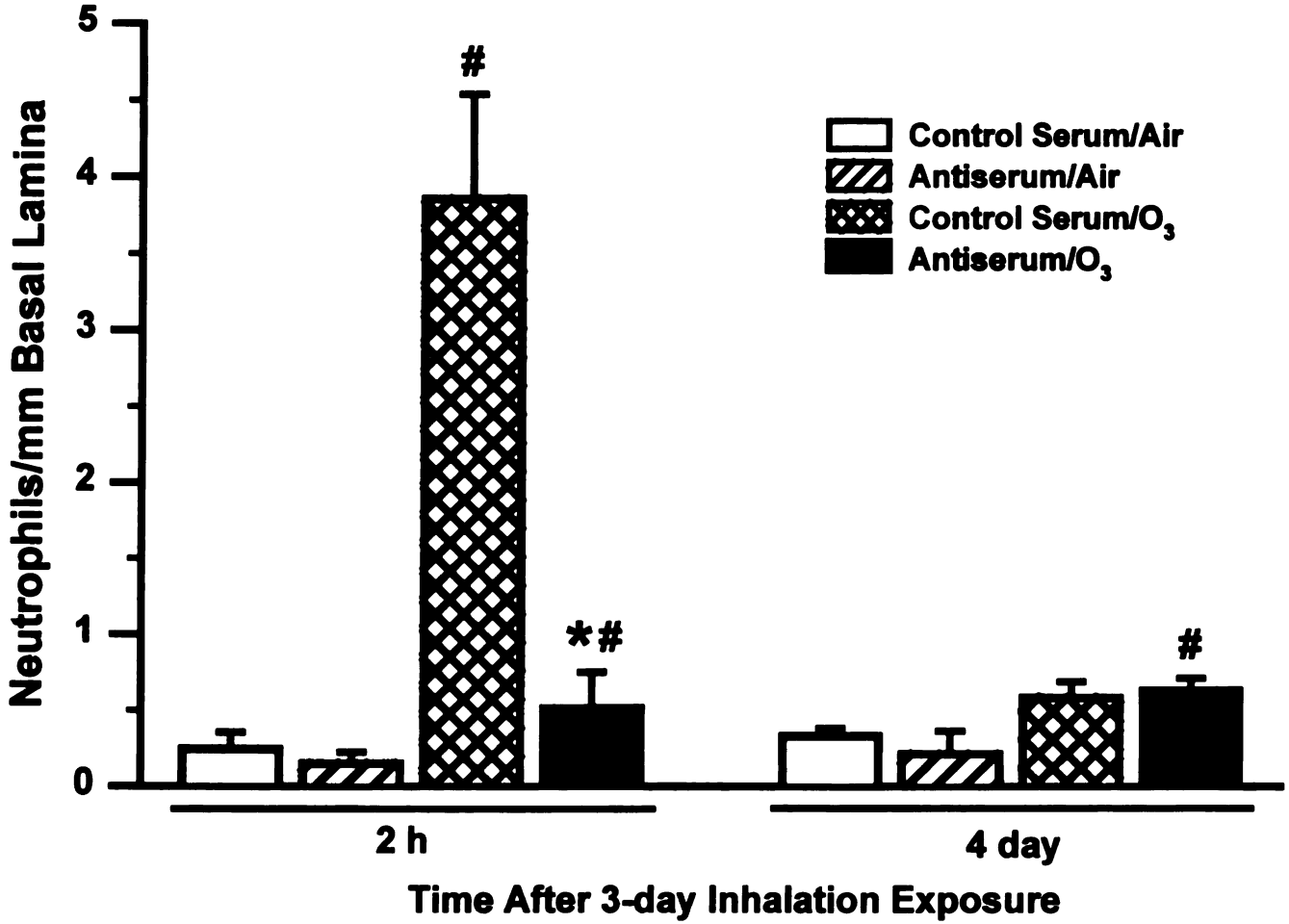

Figure 3-4. Effect of antiserum treatment on the number of intraepithelial neutrophils in the NTE. Bars represent the group mean ± SEM (n = 6/group). *Significantly different from control serum treated/ozone-exposed rats killed 2 h after exposure ($p \leq 0.05$). #Significantly different from air-exposed rats injected with the same type of serum and killed at the same time after exposure ($p \leq 0.05$).

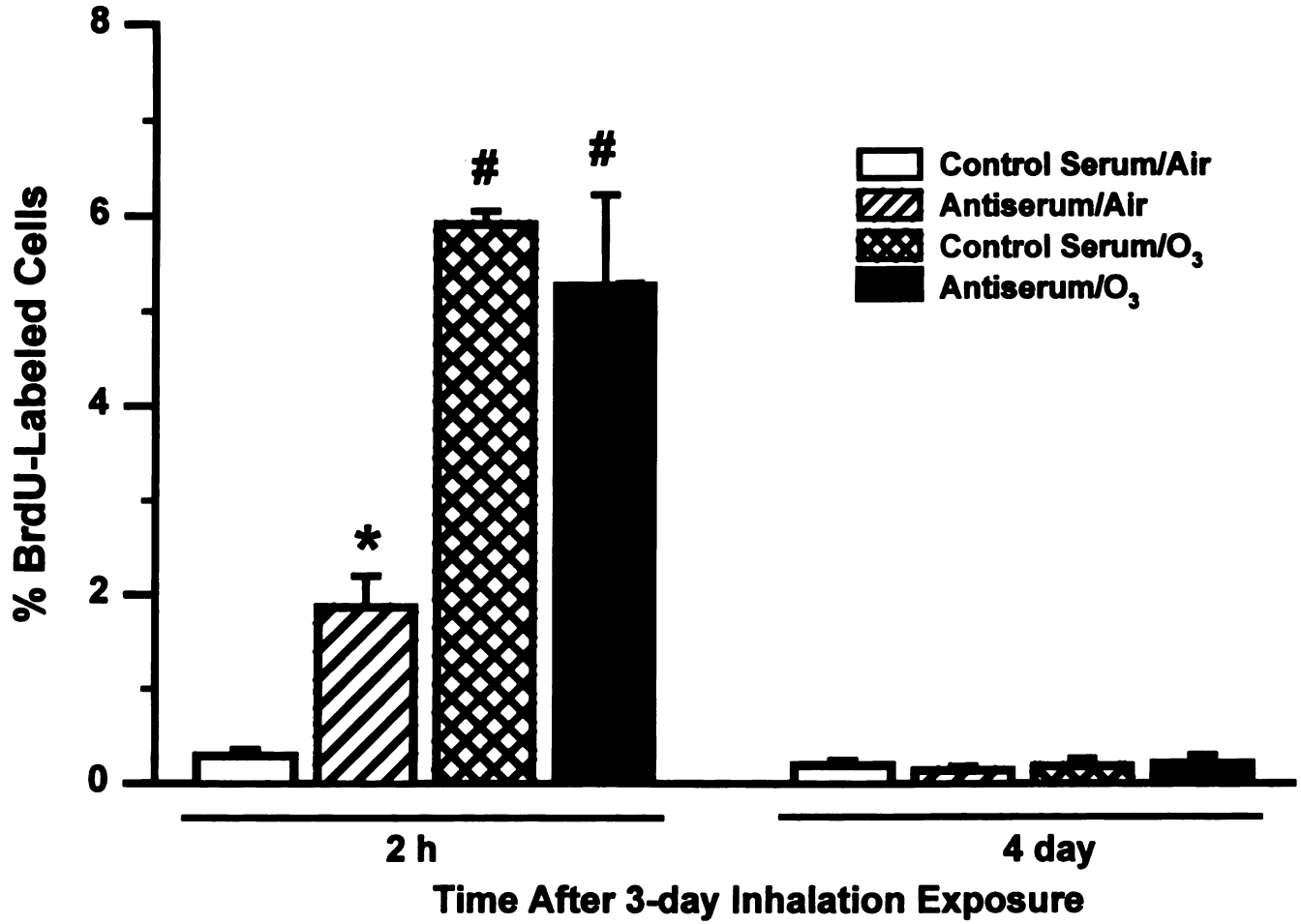

Figure 3-5. Effect of antiserum treatment on the BrdU-labeling index (LI) in the NTE. Bars represent the group mean ± SEM (n = 6/group). *Significantly different from control serum-treated/air-exposed rats killed 2 h after exposure ($p \leq 0.05$). #Significantly different from air-exposed rats injected with the same type of serum and killed at the same time after exposure ($p \leq 0.05$).

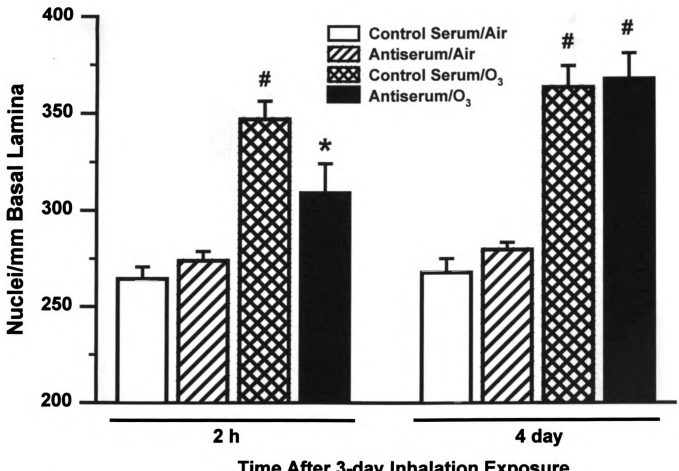

Figure 3-6. Effect of antiserum treatment on the epithelial cell numeric density in the NTE. Bars represent the group mean ± SEM (n = 6/group). *Significantly different from control serum-treated/ozone-exposed rats killed 2 h after exposure ($p \leq 0.05$). #Significantly different from air-exposed rats injected with the same type of serum and killed at the same time after exposure ($p \leq 0.05$).

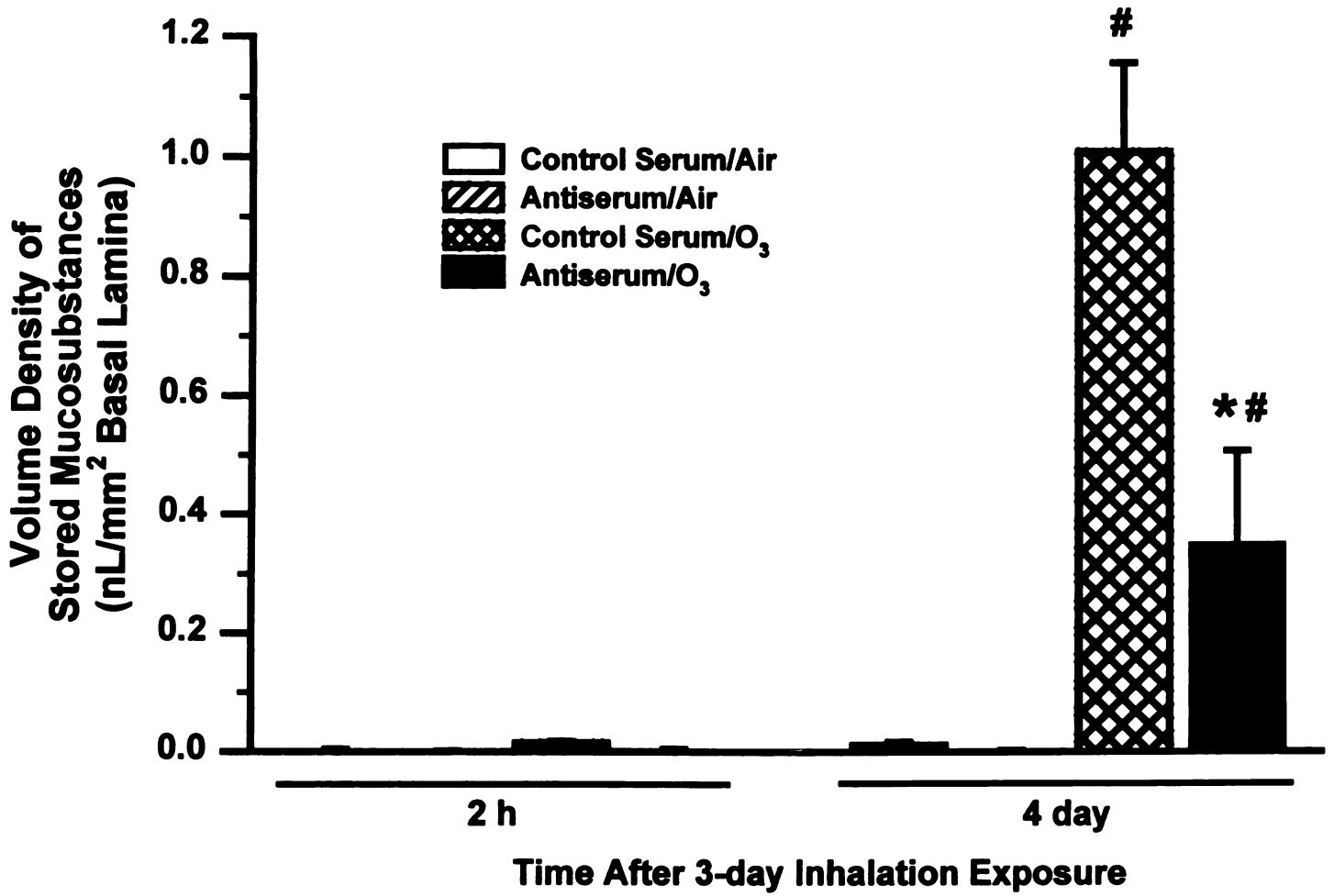

Figure 3-7. Effect of antiserum treatment on the amount of stored mucosubstances in the NTE. Bars represent the group mean ± SEM (n = 6/group). *Significantly different from control serum-treated/ozone-exposed rats killed 4 days after exposure ($p \leq 0.05$). #Significantly different from air-exposed rats injected with the same type of serum and killed 4 days after exposure ($p \leq 0.05$).

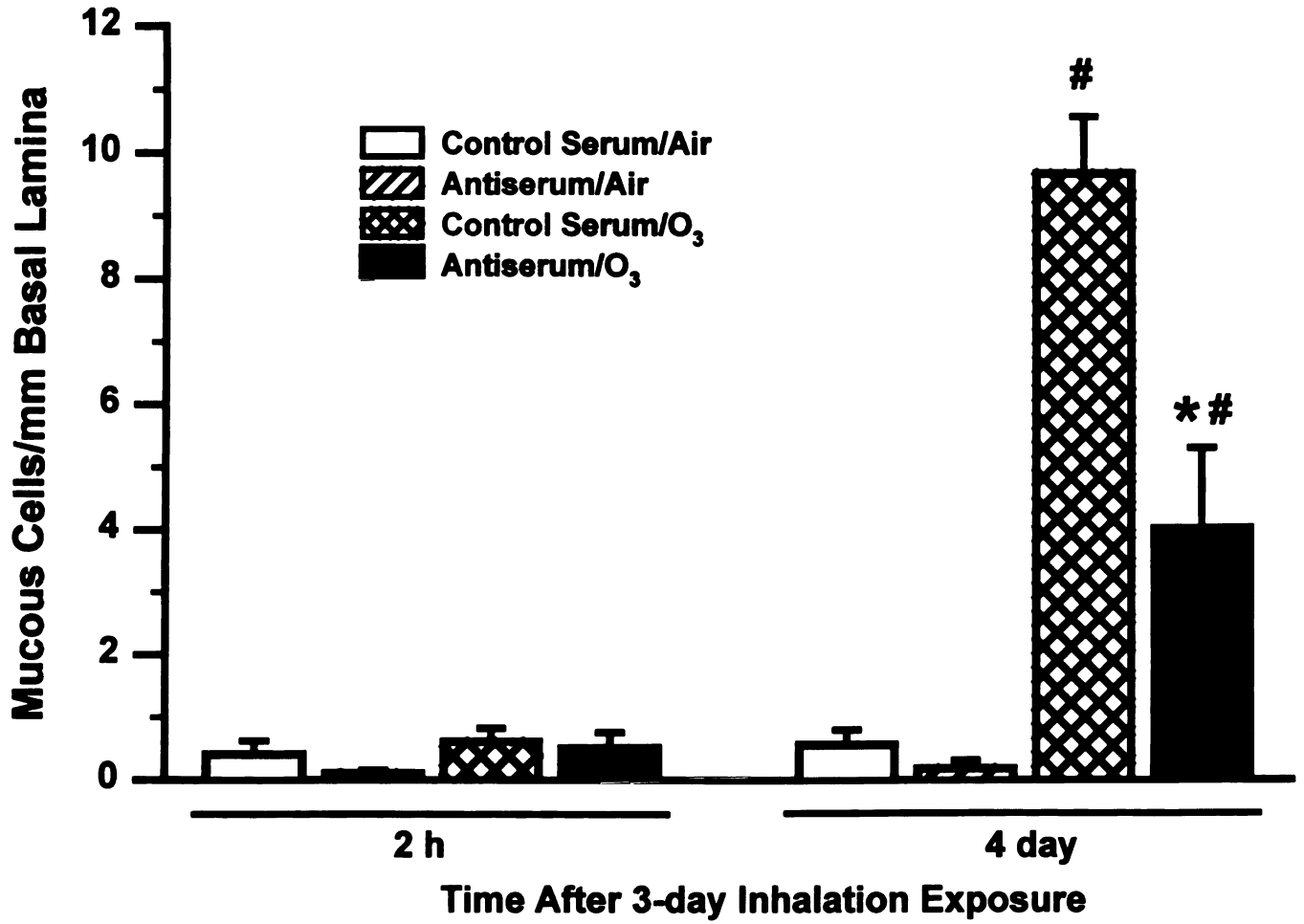

Figure 3-8. Effect of antiserum treatment on the mucous cell numeric density in the NTE. Bars represent the group mean ± SEM (n = 6/group). *Significantly different from control serum-treated/ozone-exposed rats killed 4 days after exposure ($p \leq 0.05$). #Significantly different from air-exposed rats injected with the same type of serum and killed 4 days after exposure ($p \leq 0.05$).

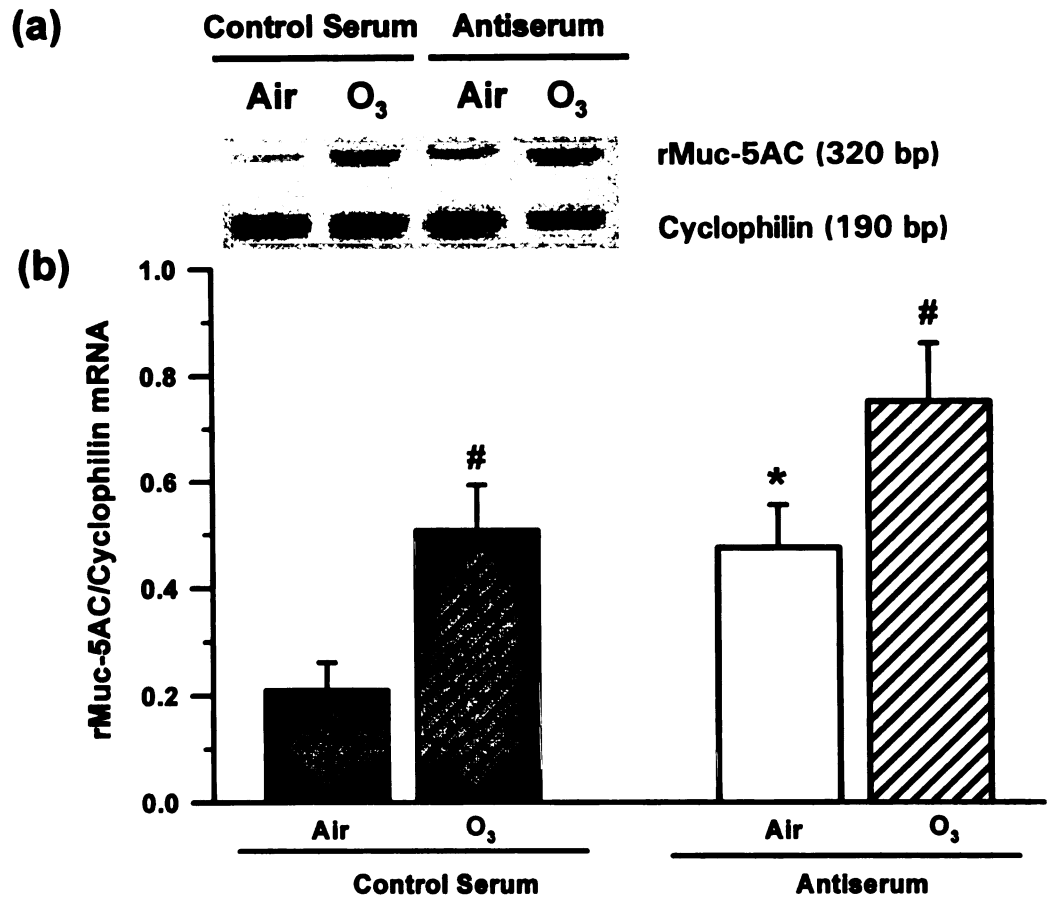

Figure 3-9. Effects of antiserum treatment on the ozone-induced rMuc-5AC mRNA upregulation in maxilloturbinates. (a) Digitized image of representative RT-PCR cDNA bands from each pooled experimental group on an agarose gel stained with ethidium bromide. cDNA products for rMuc-5AC and cyclophilin were produced by reverse transcription of RNA from maxilloturbinates and PCR-amplification using primers specific for rat Muc-5AC and cyclophilin cDNA sequences. (b) Bars represent the pooled group mean $\pm$ SEM (n = 18/ group). *Significntly different from control serum-treated/ air-exposed rats ($p \leq 0.05$). #Significantly different from air-exposed rats injected with the same type of serum ($p \leq 0.05$).

Discussion

The results of the present study indicate that ozone-induced MCM is, at least in part, neutrophil-dependent, while the induced epithelial proliferation (*i.e.*, epithelial DNA synthesis and hyperplasia) and the increase in mucin-specific (rMuc-5AC) mRNA levels are independent of the ozone-induced neutrophilic influx in the NTE of rats. To the best of our knowledge, this is the first study which reports the contribution of neutrophils in ozone-induced nasal epithelial alterations in the NTE of rats. Treatment with anti-neutrophil serum depleted the circulating pool of neutrophils and markedly attenuated the ozone-induced neutrophilic influx into the NTE (~ 90% fewer than control serum-treated/ozone-exposed rats), by 2 h after 3 days of exposure. At four days after exposure, antiserum-treated animals had 60 - 70% less ozone-induced MCM, compared to ozone-exposed rats treated with control serum. In contrast, these antiserum-treated, ozone-exposed animals still had a similar magnitude of epithelial cell proliferation, compared to that observed in control serum-treated, ozone-exposed animals killed the same postexposure time. In addition, ozone exposure induced a similar increase in rMuc-5AC mRNA in both control serum- and antiserum-treated rats.

The possible involvement of neutrophilic inflammation in the pathogenesis of ozone-induced MCM has been investigated in recent studies in our laboratory by attenuating or augmenting the inflammatory response in the nasal airways of ozone-exposed rats. In one of these studies, rats repeatedly exposed to ozone (0.5 ppm, 8 h/day for 3 or 5 days) and concurrently treated with a topical steroid, fluticasone propionate (50 μg/rat, 2

times/day by intranasal instillation), had significantly less neutrophilic inflammation and markedly attenuated MCM in the NTE than did rats exposed to ozone but intranasally instilled with saline only (Hotchkiss *et al.*, 1998). In another recent study, rats were exposed to ozone (0.5 ppm, 8 h/day for 3 days) and then intranasally instilled with a potent proinflammatory agent, bacterial endotoxin (100 μg/day for 2-consecutive days), prior to the appearance of MCM. Ozone-exposed rats instilled with endotoxin had markedly enhanced MCM in the NTE, compared to ozone-exposed, saline-instilled rats (Fanucchi *et al.*, 1998). Results of these studies, like the results of the present study, suggest that neutrophilic inflammation may play a crucial role in the pathogenesis of MCM in the NTE of ozone-exposed rats.

A causative relationship between neutrophil accumulation and abnormal increases in mucous cells has also been addressed by previous studies in distal airways of rodents. Instillation of supernatant from either lysed, purified neutrophils or activated neutrophils into the trachea of hamsters (Snider *et al.*, 1985) or rats (Lundgren *et al.*, 1988) results in an increase (50 - 300% above controls) of the number of mucous goblet cells in the tracheobronchial epithelium. The MCM or mucous cell hyperplasia induced by the neutrophil-conditioned supernatant was inhibited by an anti-inflammatory glucocorticoid, dexamethasone (Lundgren *et al.*, 1988). Anti-inflammatory drugs including dexamethasone and indomethacin have also been shown to inhibit the MCM or mucous cell hyperplasia induced by cigarette smoke which induces neutrophilic inflammation in pulmonary airways of rats (Jones and Reid, 1978; Rogers and Jeffery, 1986). Even though these previous studies suggest that inflammation is an important factor in the

development of MCM or mucous cell hyperplasia in rodent airways, little is known about the underlying mechanisms by which neutrophils contribute to in the abnormal proliferation or differentiation of airway mucous cells.

Neutrophils are a primary source of inflammatory mediators. Proteases derived from neutrophils (*i.e.*, cathepsin G, elastase) are well known mucous secretagogues in airway epithelial cells (Breuer *et al.*, 1993; Sommerhoff *et al.*, 1990). Intra-airway instillation of neutrophil elastase induces MCM in hamster airways (Breuer *et al.*, 1985, 1993; Jamil *et al.*, 1997). Elastase inhibitors (e.g., chloromethyl ketone, eglin C) as well as dexamethasone have been shown to prevent the MCM induced by neutrophil elastase (Snider *et al.*, 1985; Lundgren *et al.*, 1988). Janoff *et al.* (1979, 1983) have suggested that cigarette smoke-induced abnormal increases of mucous cells in rat pulmonary airways may be due to an inactivation of endogenous anti-proteases (e.g., α_1-antitrypsin) resulting in enhanced proteolytic activity in pulmonary airways. These findings suggest that neutrophil-derived proteases may play an important role in the pathogenesis of airway mucous overproduction and chronic obstructive airway disorders.

In addition to the proteases, neutrophils release several inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) that can stimulate other airway resident cells to release various inflammatory mediators. Recent investigations have focused on the role of inflammatory cytokines in abnormal airway mucous responses. *In vitro* studies have demonstrated that interleukin-1β (IL-1β), TNF-α, and IL-6 can cause mucin hypersecretion and/or mucin gene upregulation in airway epithelial cells (Levine *et al.*, 1994, 1995; Jarry *et al.*, 1996). Transgenic mice

overexpressing IL-4 or IL-5 have increased mucous cells (Rankin *et al.*, 1996; Jain-Vora, *et al.*, 1997; Lee *et al.*, 1997) as well as mucin hypersecretion (Temann *et al.*, 1997) in tracheobronchial and pulmonary airways. Inflammatory cytokines have been found in the bronchoalveolar or nasal lavage fluid and airway tissues of humans exposed to ozone (Calderon Garciduenas *et al.*, 1995; Koren *et al.*, 1990; Devlin, *et al.*, 1991). Certain ozone-inducible cytokines such as IL-6 have been suggested as possible mediators of cellular reparative responses in ozone-injured rat pulmonary airways by attenuating initial injury and inflammation (McKinney *et al.*, 1998). Future studies are needed to determine the role of inflammatory cytokines and other soluble mediators derived from neutrophils in the pathogenesis of ozone-induced MCM in the NTE of rats.

In the present study, we also examined the role of neutrophils in ozone-induced proliferative responses in the NTE. Our findings suggest that neutrophils do not play a role in the ozone-induced epithelial proliferation, although the onset of epithelial hyperplasia seems to be delayed in antiserum-treated rats exposed to ozone (*i.e.*, at 2 h postexposure, no significant increase of the NTE cell number was observed in these animals, compared to control serum-treated/air-exposed controls). Similar observations were made in our recent studies in which the magnitude of ozone-induced epithelial proliferation was not affected by the severity of concurrent inflammation in the NTE (Hotchkiss *et al.*, 1998; Fanucchi *et al.*, 1998). Several previous studies have demonstrated that ozone-induced epithelial injury in pulmonary airways of rats is mainly mediated, not by neutrophils, but by direct ozone toxicity (Hyde *et al.*, 1992; Pino *et al.*, 1992b; Schuller Levis *et al.*, 1994). It may be possible that ozone-induced pre-

metaplastic epithelial responses, cell necrosis and subsequent compensatory proliferation, are events that are mediated by neutrophil-independent mechanisms in the NTE.

In addition, the present study was designed to determine the involvement of neutrophilic inflammation in ozone-induced increase in mucin (rMuc-5AC) mRNA levels, a potential early molecular indicator of subsequent MCM in the NTE. The results of the present study do not support our hypothesis that neutrophils contribute to the ozone-induced elevation of mucin mRNA expression in the NTE. Several investigators have demonstrated that irritant-induced MCM in airway epithelium with accompanying increases in mucin mRNA expression is modulated at transcriptional and/or post-transcriptional levels (Jany *et al.*, 1991; Borchers and Leikauf, 1997). In addition, some inflammatory mediators have induced mucin gene upregulation as well as mucous overproduction in airway epithelial cells as described above (Voynow *et al.*, 1997; Breuer *et al.*, 1985; Temann *et al.*, 1997). Our present results, however, suggested that the upregulation of mucin mRNA, in the absence of concurrent neutrophilic inflammation, is not sufficient for the full phenotypic development of ozone-induced MCM. It seems likely that the most critical events in the pathogenesis of ozone-induced MCM is the translation of mucin mRNA or post-translational processing of the apomucin core protein (e.g., glycosylation, transport through rough endoplasmic reticulum and Golgi, storage into secretory granules) in the NTE, which may be neutrophil-dependent. In addition to the activation of biosynthesis of this characteristic functional molecule (*i.e.*, mucin) in the transformed mucous cells, neutrophils could play roles in the inhibition of mucin secretion from the mucous granules or in the architectural differentiation of mucous cells.

Though it is clear that ozone exposure resulted in a further elevation of rMuc-5AC mRNA (58%) than air exposure in antiserum-treated rats, antiserum treatment induced a 2-fold increase in rMuc-5AC mRNA levels in air-exposed control rats, compared to the basal expression of rMuc-5AC in control serum-treated/air-exposed rats. We do not know the reason, however, several possibilities are postulated. There would be mild bacterial infection in the respiratory airways of these animals due to depletion of the body's primary defense system, which may lead to induction of certain cellular genes. It may be also possible that small numbers of neutrophils which normally present in nasal tissues could play a role in maintaining the homeostasis of epithelium. Otherwise, phagocytosis of the antiserum-bound circulating neutrophils may release soluble substances which finally signal for the induction of mucin genes. We observed an unexpected increases of the epithelial DNA synthesis in the NTE of antiserum-treated, air-exposed rats at 2 h postexposure, and it seems likely due to the similar reasons.

Although neutrophil depletion did markedly attenuate the subsequent development of ozone-induced MCM in the NTE of rats, it did not completely eliminate it. This observation suggests that although neutrophilic inflammation plays a major role in development of MCM, neutrophil-independent mechanisms may also contribute to the ozone-induced MCM in rat NTE.

CHAPTER 4

Effects of Pre-Existing Rhinitis on Ozone-Induced Mucous Cell Metaplasia in Rat Nasal Airways

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Abstract

People with airway diseases may be more susceptible to the adverse effects of air pollutants than healthy subjects. Ozone causes rhinitis and nasal epithelial alterations. The toxicity of ozone on nasal airways with pre-existing rhinitis has not been investigated. The present study was designed to determine the effect of endotoxin-induced rhinitis on ozone-induced epithelial alterations, especially mucous cell metaplasia (MCM), in the nasal transitional epithelium (NTE) of rats. Six h prior to daily inhalation exposure, male F344/N rats were intranasally instilled with saline or endotoxin (100 μg/day). Rats were killed 2 h or 4 days after 3-day (8 h/day) exposure to ozone (0.5 ppm) or filtered air (0 ppm). The maxilloturbinate from one nasal passage was processed for morphometric analyses of the numbers of neutrophils and epithelial cells, and the amount of intraepithelial mucosubstances (IM) in the NTE. The maxilloturbinate from the other nasal passage was processed for a mucin-specific (rMuc-5AC) mRNA analysis. At 2 h postexposure, endotoxin/ozone-exposed rats had 48 and 3 times more neutrophils in the NTE than did saline/air- and saline/ozone-exposed rats, respectively. Ozone-exposed rats had 35% more NTE cells and 2-fold more mucin mRNA than did saline/air-exposed rats, independent of endotoxin exposure. At 4 days postexposure, endotoxin/ozone-exposed rats had 5 and 2 times more IM and mucous cells, respectively, than did saline/air- and saline/ozone-exposed rats. Though endotoxin/air-exposed rats killed at 2 h postexposure had more neutrophils (40 fold), epithelial cells (27%) and mucin mRNA (2 fold) in the NTE than did saline/air-exposed rats, no MCM was present

in those rats killed at 4 days postexposure. The results of the present study indicated that pre-existing rhinitis augments ozone-induced MCM.

Introduction

Ozone is the major oxidant gas in photochemical smog. Inhalation of ozone induces morphologic and biochemical changes in the respiratory mucosa of humans as well as laboratory animals. In F344/N rats, exposure to acute (*i.e.*, days) or chronic (*i.e.*, weeks or months) ozone (0.5 - 1.0 ppm) induces rhinitis and marked mucous cell metaplasia (MCM) in the nasal transitional epithelium (NTE) lining the lateral meatus of the proximal nasal airways of rats (Harkema *et al.*, 1997, 1989; Hotchkiss *et al.*, 1991). How ozone exposure induces MCM is unknown. However, we have recently observed that the ozone-induced MCM is, at least in part, dependent on neutrophilic inflammation (Cho *et al.*, 1998), and is markedly attenuated by an anti-inflammatory steroid, fluticasone propionate (Hotchkiss *et al.*, 1998). In addition, neutrophilic inflammation accompanies an increase of mucin-specific gene (rMuc-5AC mRNA) expression prior to the onset of the MCM in the NTE (Cho *et al.*, 1997).

Both cellular inflammation and overproduction/hypersecretion of mucus are important factors in the pathogenesis of airway diseases such as asthma, chronic bronchitis, and allergic rhinitis (Robbins *et al.*, 1984a,b; Aikawa *et al.*, 1992). Since ozone exposure also induces airway inflammation and mucous hypersecretion, it is

possible that patients suffering from chronic airway diseases may be more susceptible to the toxic effects of ozone.

Gram-negative bacterial infection (e.g., *Haemophilus influenzae*) is frequently observed in patients with chronic airway diseases (Hamacher *et al.*, 1995; Taytard *et al.*, 1995; Imundo *et al.*, 1995). Endotoxins are lipopolysaccharide-protein molecules, located in the outer cell walls of gram-negative bacteria, which are responsible for the bacteria-induced inflammatory responses (Brigham *et al.*, 1986; Reyes *et al.*, 1980). Endotoxins are potent chemotaxinogens for neutrophils (Issekutz et al., 1982; Hewett and Roth, 1993). In rats, a single intranasal instillation of endotoxin induces a transient, but conspicuous neutrophilic rhinitis (Harkema *et al.*, 1988). Though endotoxin induces DNA synthesis and epithelial proliferation in the NTE of rats (Harkema and Hotchkiss, 1993), it does not cause the MCM that is a principal feature of ozone-induced alterations in the NTE (Harkema and Hotchkiss, 1991).

The present study was designed to examine the influence of pre-existing, endotoxin-induced neutrophilic rhinitis on acute ozone-induced alterations in the NTE of rats. For this purpose, animals were treated intranasally with endotoxin prior to daily inhalation exposure to ozone. We determined the effects of endotoxin-induced rhinitis on the severity of ozone-induced MCM as well as epithelial hyperplasia and mucin mRNA upregulation in the NTE. The results of this study confirmed our hypothesis that pre-existing airway inflammation exacerbates the nasal epithelial lesions induced by ozone.

Materials and Methods

Animals and exposure. Sixty-four male F344/N rats (Harlan Sprague-Dawley, Indianapolis, IN), 10 - 12 weeks of age, were randomly assigned into one of 8 experimental groups (n = 8/group). Rats were housed two per cage in polycarbonate shoebox-type cages with Cell-Sorb Plus bedding (A&W Products, Inc., Cincinnati, OH) and filter caps. Water and food (Tek Lab 1640; Harlan Sprague Dawley, Indianapolis, IN) were available *ad libitum*. Rats were maintained on a 12-h light/dark cycle beginning at 6:00 a.m. under controlled temperature (16 - 25°C) and humidity (40 - 70%). Rats were conditioned in whole-body exposure chambers (HC-1000, Lab Products, Maywood, NJ) supplied with filtered air for 1 day prior to the start of the inhalation exposure. The rats were individually housed in rack-mounted stainless-steel wire cages with free access to food and water. The chamber temperature and relative humidity in the chamber as well as room light setting were maintained as described above.

Six h prior to daily inhalation exposure, rats were briefly anesthetized with 4% halothane in oxygen, after which 50 µl of bacterial endotoxin (lipopolysaccharide from *Pseudomonas aeruginosa* Serotype 10; Sigma Chemical Co., St. Louis, MO) in pyrogen-free saline (1 mg endotoxin/ml saline) were instilled into each nasal passage of 32 rats. The other 32 rats were treated with only pyrogen-free saline (vehicle control). We have previously reported that six h after intranasal instillation of endotoxin, rats have a marked neutrophilic rhinitis (Harkema *et al.*, 1988).

Rats instilled with saline or endotoxin were exposed to either filtered air (0 ppm, air control) or 0.5 ppm ozone in the whole-body exposure chambers, 8 h/day (10 pm - 6 am), for 3 days. Ozone was generated with an OREC Model O3VI-O ozonizer (Ozone Research and Equipment Corp., Phoenix, AZ) as explained in detail in Chapter 2. The chamber ozone concentrations (mean $\pm$ standard deviation) during the 3-day-exposures to 0.5 ppm-ozone were 0.501 $\pm$ 0.011. The chamber ozone concentrations during the 3-day-exposures to filtered air were maintained less than 0.05 ppm.

Necropsy and tissue preparation for morphometric analyses and RNA isolation. Rats were killed 2 h or 4 days after the end of 3-day inhalation exposure. Rats were deeply anesthetized using 4% halothane in oxygen and killed by exsanguination via the abdominal aorta. Immediately after death, the head of each rat was removed from the carcass. After the eyes, lower jaw, skin and musculature were removed from head, the nasal airways were opened by splitting the nose in a sagittal plane adjacent to the midline. The maxilloturbinate from one nasal passage (Figure 2-1A in page 49) was excised by microdissection, and immediately homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH). The homogenate was snap frozen in liquid nitrogen and stored at -80°C until further processed for isolation of total RNA and analysis of mucin mRNA.

The opposite nasal passage was fixed in a large volume of ice-cold 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for at least 2 h. After fixation, a portion of the maxilloturbinate in the nasal passage was microdissected from the proximal nasal airway. The dissected portion of the maxilloturbinate extended from the

level of the upper incisor tooth to the distal end of the turbinate (Figure 2-1A in page 49). The dissected tissue was decalcified with 10% EDTA in 0.2 M phosphate buffer for 7 days at 4°C with gentle shaking. The decalcified maxilloturbinates were post-fixed in 1% phosphate-buffered osmium tetraoxide (OsO_4), dehydrated in increasing ethanol concentration, and rinsed with propylene oxide. The tissues were then infiltrated with epoxy resin (Poly/Bed 812-Araldite) sequentially (50% resin in propylene oxide, overnight; 100% resin for 8 h) at room temperature, and epoxy resin was polymerized at 60°C for 2 days. Semi-thin (1 μm in thickness) tissue sections were cut from the anterior face (*i.e.,* level of the incisor tooth) of each tissue block for light microscopic analyses.

One tissue section from each block was histochemically stained with 1% toluidine blue for morphological identification of epithelial cells and infiltrated neutrophils in the NTE. Another tissue section from each block was stained with Alcian Blue (pH 2.5)/Periodic Acid-Schiff's sequence (AB/PAS) to identify acidic and neutral mucosubstances in the surface epithelium.

Morphometry of neutrophilic inflammation and epithelial cell numeric density. The NTE lining the maxilloturbinate of each animal were analyzed using image analysis and standard morphometric techniques (Hotchkiss and Harkema, 1992; Hotchkiss *et al.*, 1991). Neutrophilic inflammation (intraepithelial neutrophils/mm basal lamina) and epithelial cell numeric density (epithelial nuclei/mm of basal lamina) were determined using a Power Macintosh 7100/66 computer and the public domain image analysis software (NIH Image; written by Wayne Rasband at the U.S. National Institutes of Health

and available on the Internet at http://rsb.info.nih.gov/nih-image/) as described in detail in Chapter 2.

Morphometry of stored intraepithelial mucosubstances and mucous cells. To estimate the amount of the intraepithelial mucosubstances in NTE lining the maxilloturbinates, the volume density (Vs) of AB/PAS-stained mucosubstances was quantified using the computerized image analysis system and standard morphometric techniques as described in Chapter 2. The percentage of mucous cells (epithelial cells containing AB/PAS-stained mucosubstances) in the NTE lining the maxilloturbinates was also morphometrically determined by dividing the number of mucous cell nuclei by the total number of epithelial cell nuclei and multiplying by 100.

Analysis for mucin mRNA in maxilloturbinates. Total cellular RNA was isolated from the maxilloturbinate homogenate according to the method of Chomczynski *et al.* (1987). The RNA was then analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) with rat MUC-5AC-specific primers to determine the steady-state levels of rMuc-5AC mRNA in maxilloturbinates. Cyclophilin mRNA was used as an internal standard in this semi-quantitative RT-PCR analysis. The abundance of mucin mRNA was determined by densitometric analysis. All of these techniques are described in detail in Chapter 2.

Statistical Analyses. All data were expressed as the mean group value ± the standard error of the mean (SEM). The natural logarithms of the data were used for statistical analyses to make the variances approximately equal for all groups. The data were first analyzed using three-way analysis of variance (ANOVA) to determine the potential effects of inhalation exposure (air to ozone), intranasal instillation (saline to endotoxin), and time after inhalation exposure (2 h to 4 days). Student-Neuman-Keuls Method, an all pairwise multiple comparison procedure, was followed to identify differences in the group means. Statistical analyses were performed using a commercial statistical analysis package (SigmaStat; Jandel Scientific Software, San Rafael, CA). The level of statistical significance was set at $p \leq 0.05$.

Results

Histopathology of nasal mucosa. No exposure-related lesions were observed in the nasal mucosa of maxilloturbinates lined by NTE in saline/air-exposed rats (control) at either postexposure time. Two h after 3 days of inhalation exposure to either air or ozone, rats intranasally instilled with endotoxin had a similar degree of severe neutrophilic influx (*i.e.*, neutrophilic rhinitis) principally observed in the dorsal and medial aspect of the maxilloturbinates (Figure 4-1). The neutrophilic rhinitis was characterized by endothelial margination of neutrophils in the large capacitance vessels of the lamina propria and an influx of neutrophils in the adjacent interstitial tissues, which extended into the NTE. Saline/ozone-exposed rats had only mild-to-moderate neutrophilic inflammation at the same postexposure time. Epithelial hyperplasia was the principal morphologic alteration concurrent with the neutrophilic inflammation in the NTE of rats exposed to either endotoxin or ozone alone, or in combination. The hyperplastic NTE was approximately 3 - 4 cells in thickness, compared to 1 - 2 cells in the NTE of the saline/air-exposed control rats. There were no recognizable differences in the severity of the epithelial hyperplasia among the rats exposed to either endotoxin or ozone alone, or in combination 2 h after the end of the inhalation exposure.

There was a marked MCM, characterized by the appearance of AB/PAS-stained mucous cells in the NTE of rats killed 4 days after 3 days of ozone exposure, regardless of the type of nasal instillation (*i.e.*, saline or endotoxin) (Figure 4-2). However, the magnitude of the metaplastic response was greater in rats exposed to both endotoxin and ozone. In the rats containing MCM, the majority of the mucous cells were present in the

dorsal and medial aspects of the NTE lining the maxilloturbinates. Epithelial hyperplasia persisted with the MCM in those rats, but the magnitude was less severe than that observed in the similarly exposed rats killed 2 h after inhalation exposure. Endotoxin/air-exposed rats had only occasional isolated mucous cells in the NTE at 4 days after the inhalation exposure. In addition, epithelial hyperplasia was not observed in these rats killed at this time point. Neutrophils were rarely present in the turbinate mucosa of rats killed 4 days after inhalation exposure.

Neutrophilic inflammation. Exposure to either ozone or endotoxin alone, or to both agents, resulted in a transient influx of neutrophils in the NTE lining the maxilloturbinates (Figure 4-3). Two h after 3 days of inhalation exposure, there were 40-, 8- and 48-times more neutrophils in the NTE of endotoxin/air-exposed, saline/ozone-exposed and endotoxin/ozone-exposed rats, respectively, compared to those in saline/air-exposed controls. Rats exposed to both endotoxin and ozone had significantly more (3-times) neutrophils in the NTE than did saline/ozone-exposed rats. At 4 days after 3 days of inhalation exposure, there were no significant differences in the number of intraepithelial neutrophils in rats of all exposure groups.

Epithelial cell numeric density. Rats exposed to either ozone or endotoxin, or both, had increased numbers of NTE cells lining the maxilloturbinates, compared to saline/air-exposed controls (Figure 4-4). At 2 h after the end of the inhalation exposure, epithelial cell numeric densities in endotoxin/air-exposed, saline/ozone-exposed, and endotoxin/ozone-exposed rats were 28 - 37% greater than those in saline/air-exposed

controls. No significant differences were observed in the number of NTE cells of rats in these three exposure groups. At 4 days after the inhalation exposure, only rats exposed to ozone had more epithelial cells in the NTE (12 - 17%) than did saline/air-exposed controls, independent of endotoxin instillation. However, these saline/ozone-exposed and endotoxin/ozone-exposed rats killed 4 days after inhalation exposure had significantly fewer epithelial cells in the NTE (14% and 9%, respectively), compared to the similarly-exposed rats killed 2 h after inhalation exposure.

Stored intraepithelial mucosubstances. At 4 days after the end of the inhalation exposure, saline/ozone-exposed and endotoxin/ozone-exposed rats had significantly more intraepithelial mucosubstances (6- and 12- times, respectively), compared to air/saline-exposed controls (Figure 4-5). The amounts of intraepithelial mucosubstances in the NTE of endotoxin/ozone-exposed rats was 2-fold greater than those of saline/ozone-exposed rats. There was no significant difference in the volume densities of intraepithelial mucosubstances between endotoxin/air-exposed and the saline/air-exposed rats. Only scant amounts of AB/PAS-positive mucosubstances were present in the NTE of rats killed 2 h after the inhalation exposure.

Number of mucous cells. The percentage of mucous cells was significantly increased (5- and 10-times) in the NTE of saline/ozone-exposed or endotoxin/ozone-exposed rats, respectively, compared to that of saline/air-exposed controls (Figure 4-6). Rats exposed to both endotoxin and ozone had 2-fold more mucous cells in the NTE than did the saline/ozone-exposed rats. There was no significant difference in the number of mucous

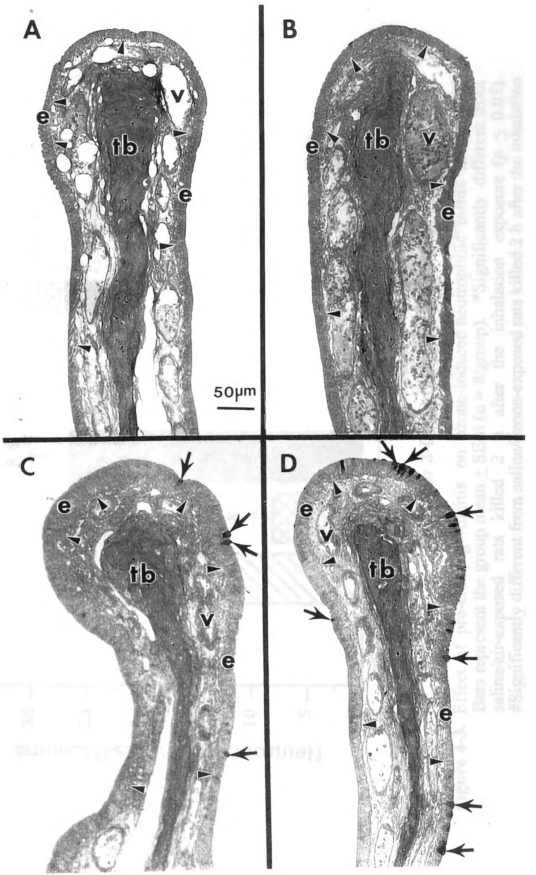
cells between endotoxin/air-exposed and saline/air-exposed rats. Few mucous cells were present in the NTE of all the rats killed 2 h after the end of the inhalation exposure.

Mucin (rMuc-5AC) mRNA expression. Exposure to either ozone or endotoxin alone, or in combination, resulted in marked increases of rMuc-5AC mRNA expression in the maxilloturbinates 2 h after the inhalation exposure (Figures 4-7 and 4-8). There were approximately 2-fold increases in rMuc-5AC mRNA levels in endotoxin/air-exposed, saline/ozone-exposed, and endotoxin/ozone-exposed rats, compared to those in the saline/air-exposed controls. No significant differences in mucin mRNA levels of maxilloturbinates were present among those rats exposed to ozone and/or endotoxin. The rMuc-5AC mRNA levels remained elevated in the maxilloturbinates of endotoxin/air-exposed rats killed 4 days after the inhalation exposure. However, mucin mRNA levels in maxilloturbinates of ozone-exposed rats (regardless of the type of nasal instillation) were not significantly different from those of saline/air-exposed controls at 4 days after the inhalation exposure.

Figure 4-1. Light photomicrographs of maxilloturbinates from rats exposed to (A) saline and filtered air (0-ppm ozone), (B) 100-μg endotoxin and filtered air, (C) saline and 0.5-ppm ozone, or (D) 100-μg endotoxin and 0.5-ppm ozone and killed 2 h after the end of the 3-day inhalation exposure. Tissues were stained with toluidine blue. Arrowheads = basal lamina between epithelium and lamina propria; arrows = neutrophils; e = epithelium (NTE); tb = turbinate bone; v = blood vessel.

A
B
C
D
rib
e
50µm

Figure 4-2. Light photomicrographs of maxilloturbinates from rats exposed to (A) saline and filtered air (0-ppm ozone), (B) 100-μg endotoxin and filtered air, (C) saline and 0.5-ppm ozone, or (D) 100-μg endotoxin and 0.5-ppm ozone and killed 4 days after the end of the 3-day inhalation exposure. Tissues were stained with AB/PAS to detect acidic and neutral mucosubstances. Arrowheads = basal lamina between epithelium and lamina propria; arrow = AB/PAS-stained intraepithelial mucosubstance in mucous cell; e = epithelium (NTE); tb = turbinate bone; v = blood vessel.


A
B
C
D
e
v
tb
50μm

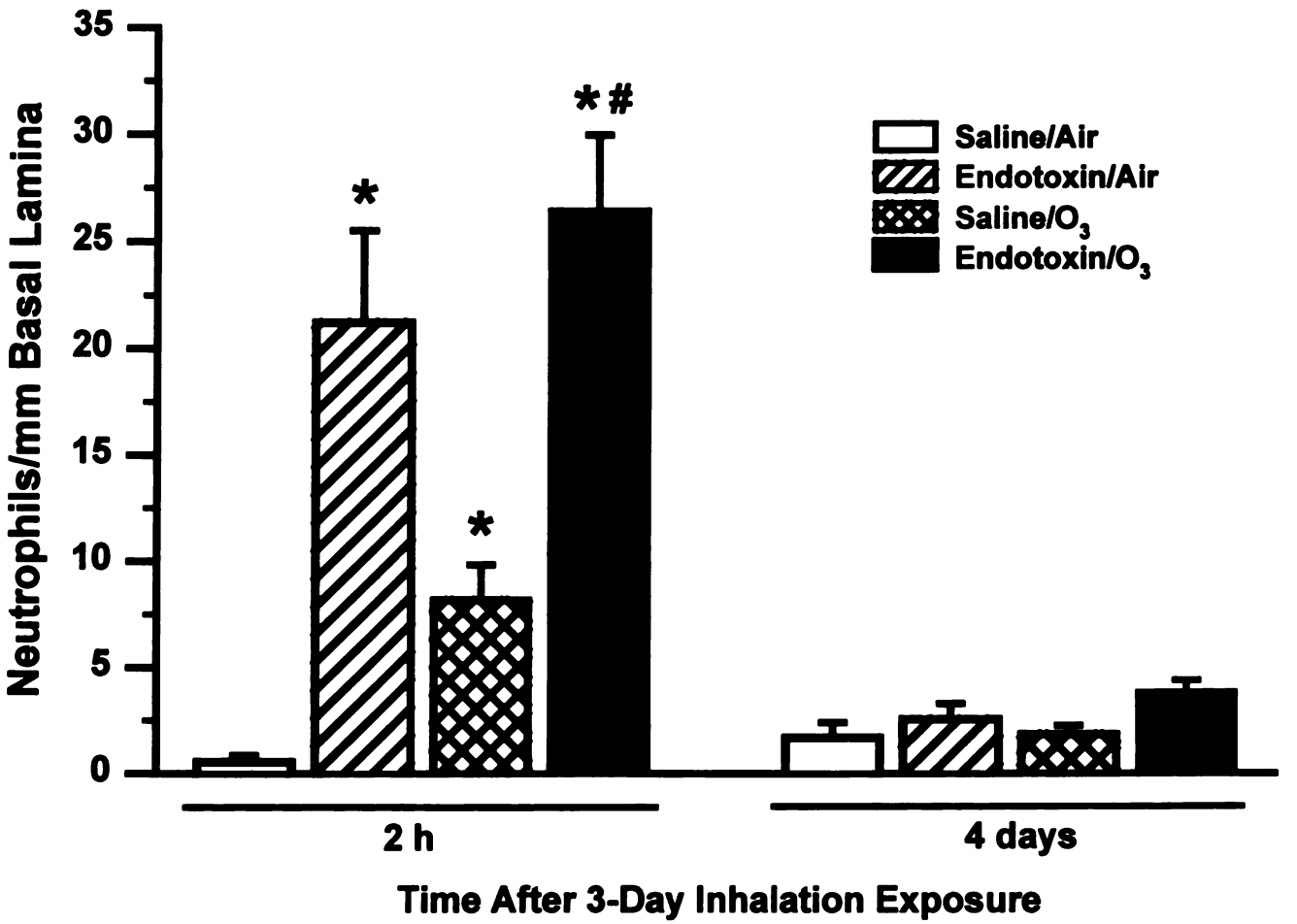


Figure 4-3. Effect of pre-existing rhinitis on ozone-induced neutrophilic influx in the NTE. Bars represent the group mean ± SEM (n = 8/group). *Significantly different from saline/air-exposed rats killed 2 h after the inhalation exposure ($p \leq 0.05$). #Significantly different from saline/ozone-exposed rats killed 2 h after the inhalation exposure ($p \leq 0.05$).

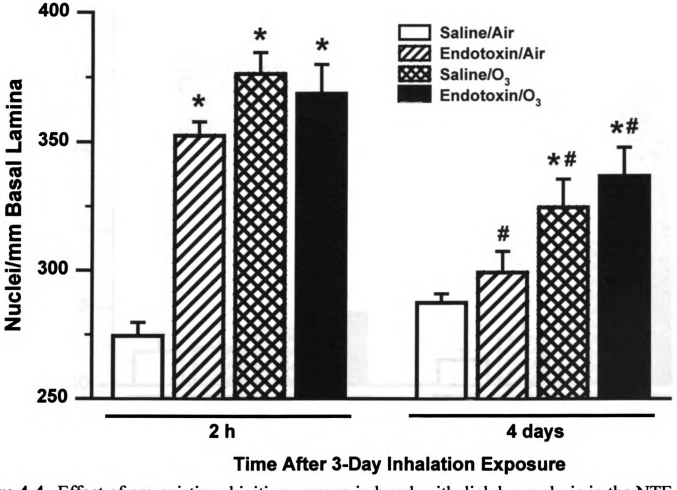


Figure 4-4. Effect of pre-existing rhinitis on ozone-induced epithelial hyperplasia in the NTE. Bars represent the group mean ± SEM (n = 8/group). *Significantly different from saline/air-exposed rats killed at the same time points ($p \leq 0.05$). #Significantly different from similarly exposed rats killed 2 h after the inhalation exposure ($p \leq 0.05$).

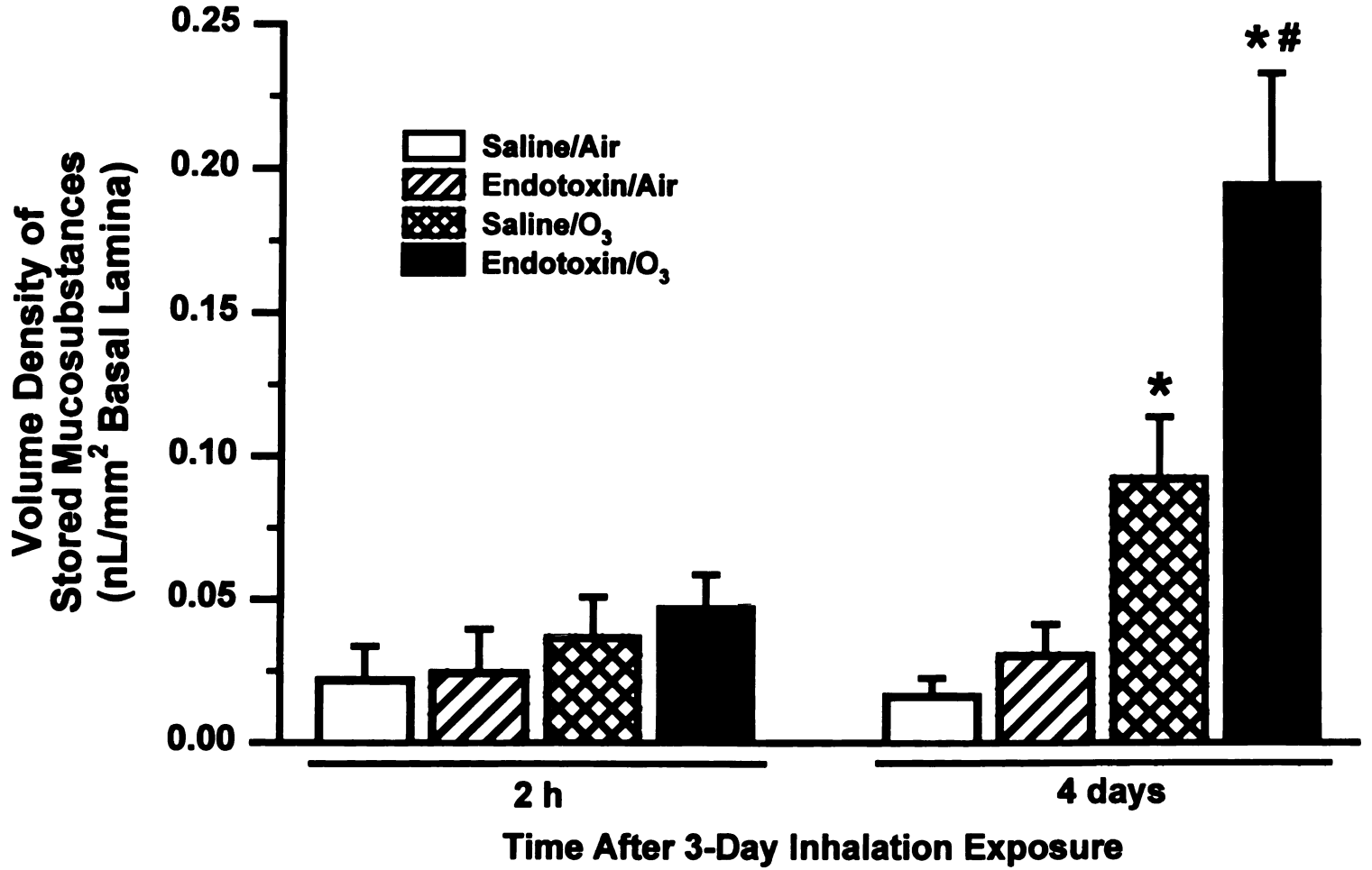


Figure 4-5. Effect of pre-existing rhinitis on ozone-induced increase in the stored intraepithelial mucosubstances in the NTE. Bars represent the group mean ± SEM (n = 8/group). *Significantly different from saline/air-exposed rats killed 4 days after the inhalation exposure ($p \leq 0.05$). #Significantly different from saline/ozone-exposed rats killed 4 days after the inhalation exposure ($p \leq 0.05$).

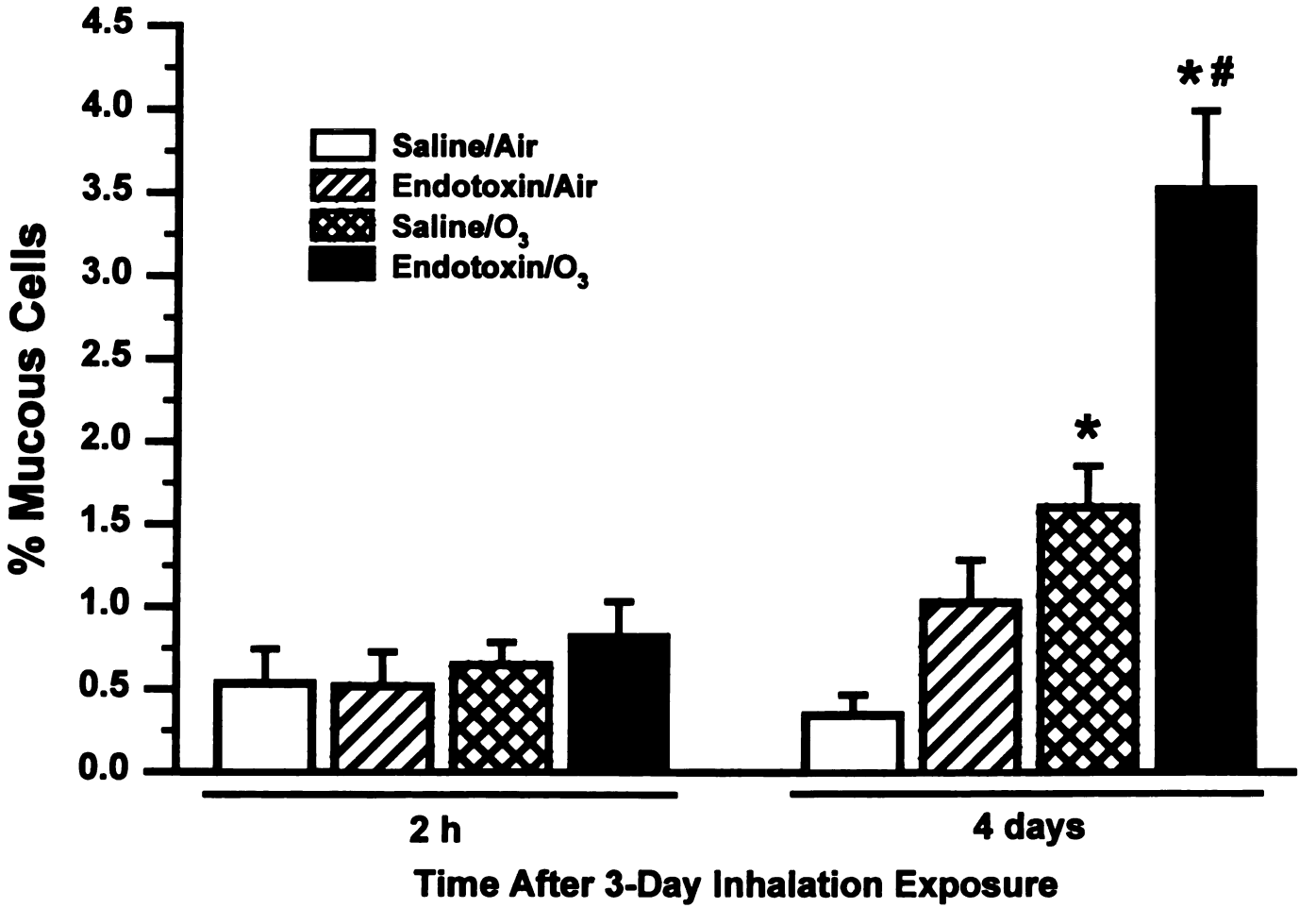


Figure 4-6. Effect of pre-existing rhinitis on ozone-induced increase in the mucous cells in the NTE. Bars represent the group mean ± SEM (n = 8/group). *Significantly different from saline/air-exposed rats killed 4 days after the inhalation exposure ($p \leq 0.05$). #Significantly different from saline/ozone-exposed rats killed 4 days after the inhalation exposure ($p \leq 0.05$).

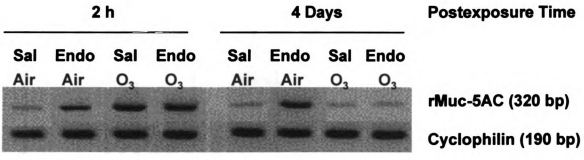


Figure 4-7. Digitized image of an ethidium bromide-stained gel with representative RT-PCR cDNA bands for rMuc-5AC and cyclophilin from each exposure group (n = 8/group). cDNA was produced by reverse transcription of RNA extracted from one maxilloturbinate of each rat and PCR-amplification using primers specific for rat MUC-5AC and cyclophilin cDNA sequences. Sal = saline; Endo = endotoxin.

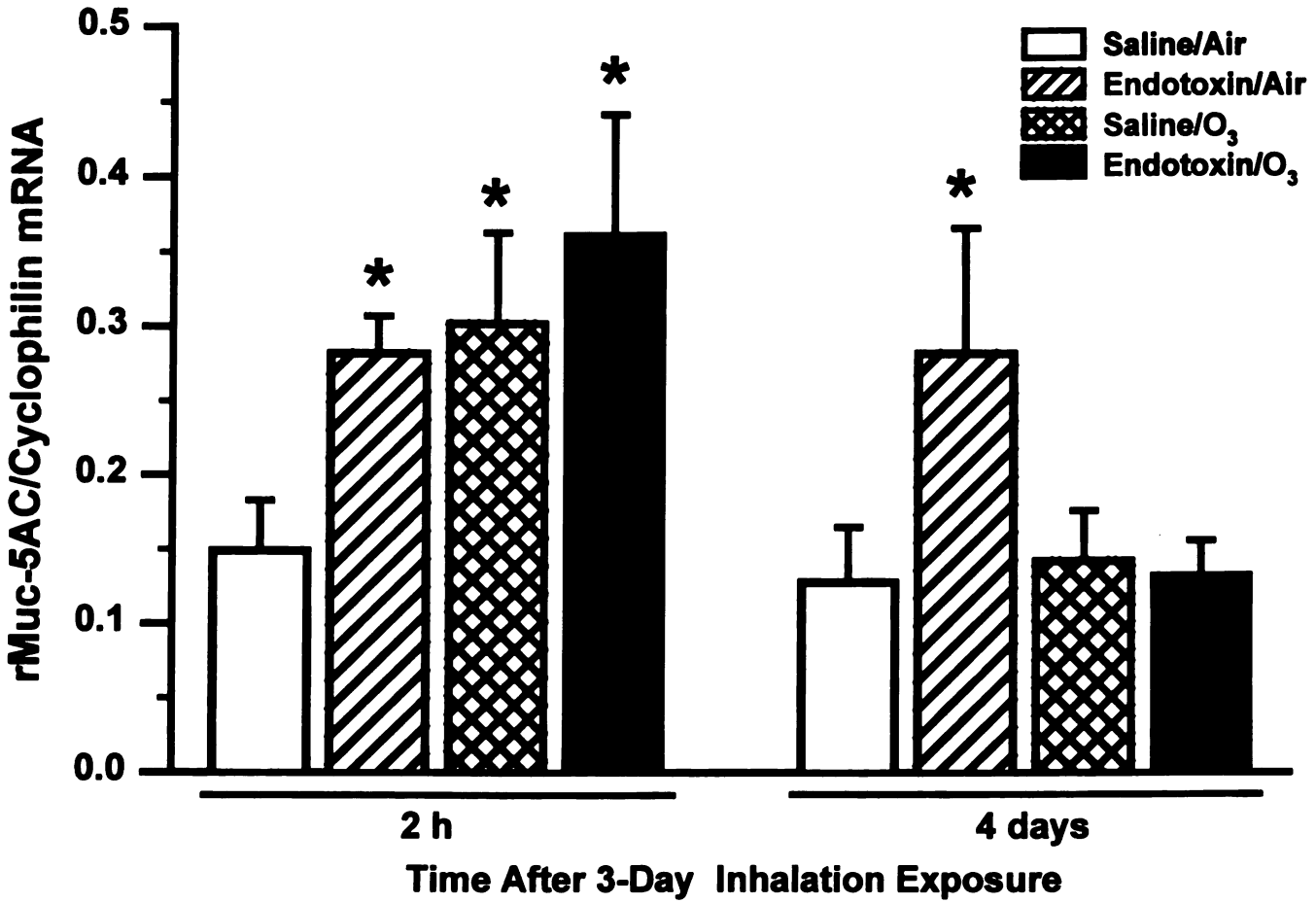


Figure 4-8. Effect of pre-existing rhinitis on ozone-induced rMuc-5AC mRNA upregulation in maxilloturbinates. Bars represent the group mean $\pm$ SEM (n = 8/group). *Significantly different from saline/air-exposed rats killed at the same time points ($p \leq 0.05$).

Discussion

The results presented in this study demonstrate that preexisting nasal inflammation (*i.e.*, neutrophilic rhinitis caused by endotoxin) can augment ozone-induced MCM in the NTE of rats. There were 2-fold greater amounts of mucosubstances and numbers of mucous cells in the NTE of rats intranasally instilled with endotoxin and exposed to ozone for 3 days, compared to those in rats instilled with saline and exposed to ozone.

A few previous studies have suggested that pre-existing airway inflammation enhances the pulmonary toxicity of inhaled pollutants (e.g., nitrogen dioxide, sulfur dioxide, or automotive emission) in laboratory animals (Gilmour, 1995; Menzel, 1994; Hubbard *et al.*, 1994; Mauderly *et al.*, 1989). The present study is the only animal study to our knowledge that reports the effects of pre-existing airway inflammation on ozone-induced nasal toxicity.

Several investigators have demonstrated that people with chronic respiratory diseases have increased adverse respiratory effects from ozone exposure. Asthmatics exposed to acute ozone (0.2 - 0.4 ppm, 2 - 6 h) have greater increases in airway obstruction and inflammation, and bronchial hyperresponsiveness as well as further decrements in lung function, compared to non-asthmatics (Basha *et al.*, 1994; Kreit *et al.*, 1989; Koenig, 1995; Scannell *et al.*, 1996). Patients with allergic rhinitis also have enhanced nasal inflammation and bronchial allergen responsiveness after exposure to ozone (0.25 - 0.5 ppm, 3 - 4 h), compared to ozone-exposed healthy subjects (Bascom *et al.*, 1990; Jorres *et al.*, 1996). In addition, epidemiologic studies have suggested that chronic ozone

exposure in many urban areas may account for the increased incidence of asthma-related summertime hospital admissions (Cody *et al.*, 1992; Dockery *et al.*, 1989).

Specific cellular and molecular mechanisms underlying ozone-induced MCM are unknown. However, the presence of copious neutrophils in the NTE before the histologic appearance of MCM have led us to hypothesize that neutrophilic inflammation plays a role in the development of ozone-induced MCM. This hypothesis was supported by the results from our most recent study in which intranasal treatment with an anti-inflammatory steroid, fluticasone propionate (100 μg/day) resulted in a marked attenuation of acute (0.5 ppm, 8 h/day for 3 days) ozone-induced neutrophilic inflammation and MCM in the NTE of rats (Hotchkiss *et al.*, 1998). In addition, we have also reported that depletion of circulating neutrophils significantly attenuated the MCM in the NTE of rats exposed to ozone (0.5 ppm, 8 h/day for 3 days) (Cho *et al.*, 1998). In the present study, we observed that endotoxin alone did not induce MCM in the NTE, but it markedly magnified the ozone-induced MCM. This suggests that the potentiating effects of endotoxin exposure on ozone-induced MCM may be mediated, not by direct effects of endotoxin on airway epithelium, but by endotoxin-induced neutrophilic inflammation.

In another recent study, we have addressed the effects of endotoxin exposure following ozone exposure on nasal airway lesions in rat NTE (Fanucchi *et al.*, 1998). In that study, rats were consecutively exposed to ozone for 3 days (0.5 ppm, 8 h/day), and then intranasally instilled with endotoxin (100 μg/day) for 2 days. The endotoxin-induced neutrophilic rhinitis markedly magnified the MCM induced by the previous

ozone exposure in the NTE. From our present and previous studies, it is postulated that neutrophilic inflammation plays an important role in the pathogenesis of MCM, and thus, neutrophilic rhinitis present in the NTE, either prior to or simultaneously with the onset of MCM, can amplify the mucous metaplastic responses to ozone.

The role of inflammatory mediators derived from neutrophils in the pathogenesis of MCM or secretory cell hyperplasia has been investigated in rodent tracheobronchial airways by others. Instillation of supernatants from either lysed purified neutrophils or activated neutrophils into the trachea of hamsters (Snider *et al.*, 1985) or rats (Lundgren *et al.*, 1988) results in an increase (50 - 300% above controls) in the number of mucous goblet cells in the tracheobronchial epithelium. MCM induced by the neutrophil-conditioned supernatant was inhibited by an anti-inflammatory steroid, dexamethasone (Lundgren *et al.*, 1988). Intra-airway instillation of neutrophil elastase induces MCM in hamster airways (Breuer *et al.*, 1993, 1985), and elastase inhibitors (e.g., chloromethyl ketone) as well as dexamethasone have been shown to prevent the MCM induced by neutrophil elastase (Breuer *et al.*, 1985; Lundgren *et al.*, 1988).

In the present study, we also determined the effect of endotoxin-induced rhinitis on ozone-induced epithelial proliferation. Pre-existing rhinitis did not alter the severity of ozone-induced epithelial cell hyperplasia in the NTE. This result was consistent with our recent findings that attenuation of neutrophilic inflammation in the NTE by depletion of circulating neutrophils (Cho *et al.*, 1998) or by intranasal instillation of an anti-inflammatory steroid (fluticasone propionate) (Hotchkiss *et al.*, 1998) did not alter the severity of the ozone-induced NTE cell proliferation. The results suggest that ozone

directly induces epithelial proliferation, independent of the preceding or concurrent inflammatory response.

The present study was also designed to determine the effects of preexisting neutrophilic rhinitis on ozone-induced mucin mRNA upregulation in maxilloturbinates. Rats exposed to ozone had a similar magnitude of rMuc-5AC mRNA expression in the nasal tissues, regardless of endotoxin instillation, at 2 h after the end of last exposure. The elevated mRNA levels did not persist with MCM at 4 days after the end of last exposure. We also observed that rats repeatedly exposed to endotoxin had an increased mucin mRNA expression in the nasal tissues without significant histologic evidence of MCM, as seen in another study (Fanucchi *et al.*, 1998). The results of our study indicate that pre-existing neutrophilic inflammation does not magnify the ozone-induced mucin mRNA upregulation. In addition, the elevated rMuc-5AC mRNA levels in the nasal tissues may not accurately predict or reflect the magnitude of the production of mucous glycoprotein in the NTE.

In conclusion, pre-existing rhinitis augmented the ozone-induced MCM in the NTE. Endotoxin exposure, alone, did not induce MCM in the NTE. This augmentation may be mediated by the intraepithelial influx of neutrophils induced by the inflammatory agent, endotoxin. The results from this airway study suggest that people with rhinitis may be more susceptible to some, but not all, of the epithelial lesions caused by exposure to high ambient concentrations of ozone.

CHAPTER 5

Ozone-Inducible Cytokines and Their Role in Mucin Gene Expression in Rat Nasal Tissues

Introduction

In our previous studies, we demonstrated that acute ozone exposure induced mucin mRNA upregulation in nasal tissues within a few hours after the start of exposure. It preceded the onset of mucous cell metaplasia (MCM) by several days and persisted with MCM. Ozone-induced MCM appears to be a multi-stage process. Ozone alone induces premetaplastic events including epithelial cell injury and proliferation and induction of mucin gene expression prior to the development of MCM. However, full phenotypic expression of MCM is dependent on the presence of inflammatory cells (*i.e.*, neutrophils). Although the results from our studies are not enough to elucidate the mechanisms of ozone-induced MCM or the dependency of MCM on the activation of mucin genes, and mucin mRNA levels in nasal tissues do not always predict the histologic appearance of mucin glycoproteins in the NTE, the increases in the steady-state levels of mucin mRNA precede the onset of the ozone-induced MCM.

Little is known about regulatory mechanisms of mucin gene expression in healthy and diseased airway epithelium. A few recent studies have reported that cytokines including TNF-α (Levine *et al.*, 1995), IL-6 (Levine *et al.*, 1994) and IL-4 (Temann *et al.*, 1997; Rankin *et al.*, 1996), or neutrophil elastases (Voynow *et al.*, 1997) induce mucin mRNA upregulation in pulmonary epithelium or in cultured airway tissues or cells. Some of these factors are also known to induce MCM and/or mucin hypersecretion in rodent airways or in cultured airway cells (Jamil *et al.*, 1997; Rankin *et al.*, 1996; Levine *et al.*, 1994, 1995). Elevated TNF-α or IL-6 levels have been correlated clinically with

airway obstructive disorders such as asthma which is characterized by mucous hypersecretion and overproduction (Marini *et al.*, 1992; Broide *et al.*, 1992)

Inhalation exposure of humans to ozone (0.1 - 0.4 ppm, 1 - 24 h) induces the release of various cytokines (e.g., TNF-α, IL-1, IL-6, IL-8) into respiratory airways (Devlin *et al.*, 1991, 1996; Koren *et al.*, 1989; Pendino *et al.*, 1994; Rusznak *et al.*, 1996). Among them, TNF-α and IL-6 are multifunctional proinflammatory cytokines, which are thought to be markers of inflammation, tissue injury and repair in ozone-exposed airways (Leikauf *et al.*, 1995; Pendino *et al.*, 1994, 1995). Both TNF-α and IL-6 are expressed by airway epithelial cells after ozone exposure in the absence of inflammatory cells (Devlin *et al.*, 1994; Beck *et al.*, 1994). However, the specific roles these cytokines play in ozone-induced airway epithelial alterations have not been investigated. In addition, very little is known about the time course of expression of these mediators in laboratory animals following exposure to ozone.

The present studies were designed to test the hypotheses that (1) acute ozone exposure causes TNF-α and IL-6 expression in nasal airways of rats, and that (2) these soluble mediators can induce mucin gene expression in rat nasal tissues. We first examined the time-dependent changes in the steady-state levels of TNF-α and IL-6 mRNAs in nasal airways during and after *in vivo* ozone exposure. Next, we examined the potential role of soluble forms of these cytokines on mucin (rMuc-5AC) mRNA expression in normal nasal tissues *in vitro*. Microdissected maxilloturbinates maintained in culture at an air-liquid interface were exposed to these cytokines to investigate the

direct effects of TNF-α or IL-6 on the expression of rMuc-5AC mRNA. A quantitative RT-PCR assay was used to evaluate the expression of rMuc-5AC mRNA in nasal tissues.

STUDY 1: TIME-DEPENDENT CHANGES OF TNF-α AND IL-6 mRNA LEVELS IN NASAL AIRWAYS AFTER SINGLE AND REPEATED OZONE EXPOSURE

Materials and Methods

Animal, exposure, sacrifice and RT-PCR analysis. RNA samples from the study described in Chapter 2 (n = 8/group) were also used for this study. In brief, total RNA was isolated from microdissected maxilloturbinates of rats exposed to 0 ppm ozone (filtered air) for 7 days, or rats exposed to 0.5 ppm ozone for 1 day or 2 days (8 h/day) and killed 2 h after the end of exposure, or rats exposed for 3 days and killed 2 h or 1, 2, and 4 days after the end of exposure.

The abundance of mRNA for each cytokine was determined by semi-quantitative RT-PCR and densitometric analysis as described in Chapter 2 using cyclophilin mRNA as an internal standard. The PCR primers specific for rat TNF-α and IL-6 cDNA sequences were synthesized and purified by the Macromolecular Structure Facility at Michigan State University. The sequences of rat TNF-α forward and reverse primers for PCR amplification were 5'-ATG AGC ACA GAA AGC ATG ATC-3' and 5'-TAC AGG CTT GTC ACT CGA ATT-3', respectively, and the predicted size of TNF-α cDNA

products was 276 bp (Nanji *et al.*, 1995). The sequences of rat IL-6 forward and reverse primers were 5'-CTT CCC TAC TTC ACA AGT C-3' and 5'-CTC CAT TAG GAG AGC ATT G-3', respectively, and the predicted size of IL-6 cDNA products was 476 bp (Farges *et al.*, 1995). PCR amplification of TNF-α cDNA started with 3 min incubation at 94°C followed by 33-cycles of a three-step temperature cycle; denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. PCR amplification of IL-6 cDNA started with 3 min incubation at 95°C followed by denaturation at 95°C for 30 sec, annealing at 56°C for 1 min and extension at 72°C for 1 min for 30 cycles. Each amplification reaction was finished with a final extension step at 72°C for 10 min. Cyclophilin mRNA was separately reverse transcribed and PCR-amplified following the three-step cycle (23 cycles, 95°C for 30 sec - 56°C for 1 min - 72°C for 1 min) using cyclophilin forward and reverse primers described in Chapter 2. The densitometric volume of the TNF-α and IL-6 cDNA bands on an agarose gel was divided by that of the cyclophilin cDNA band achieved from the same RNA sample.

Statistical analyses. Data were expressed as the mean group value ± the standard error of the mean (SEM). Data presenting the changes of TNF-α mRNA and IL-6 mRNA abundance were natural log- and square root-transformed, respectively, to make variances homogeneous. The data were analyzed for potential effects of exposure duration on cytokine mRNA expression using one-way ANOVA. Significant differences among the experimental groups were determined by use of Dunnett's Method ($p \leq 0.05$).

Results

Ozone-induced TNF-α mRNA expression. The mean steady-state level of TNF-α mRNA was increased by 70%, compared to the air-exposed controls, in rats killed 2 h after 1 and 2 days of ozone exposure (Figure 5-1). Two h following the third day of exposure, steady-state levels of TNF-α message were 110% greater than controls. The elevated levels of TNF-α mRNA significantly decreased in ozone-exposed rats killed 1, 2, and 4 days after 3 days of exposure.

Ozone-induced IL-6 mRNA expression. Ozone exposure induced an increase in IL-6 mRNA expression in nasal tissues (Figure 5-2). A significant elevation of IL-6 mRNA level was observed in rats killed 2 h after 1 day of ozone exposure (9-fold increase, compared to air-exposed controls). However, there was no difference in the nasal IL-6 mRNA levels between the control rats and the rats exposed to ozone for 2 or 3 days, regardless of the sacrificed time.

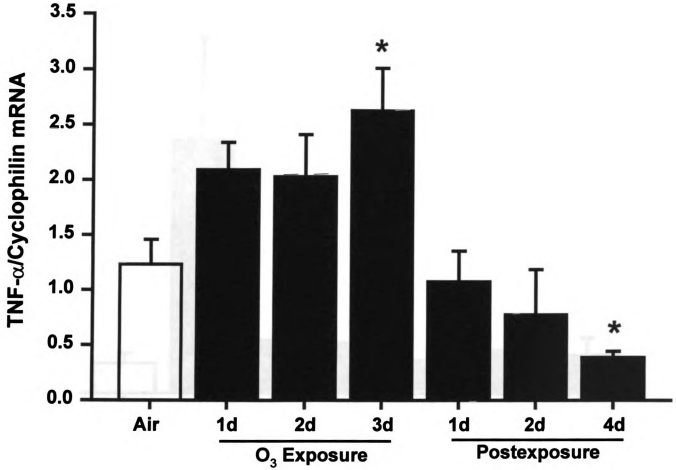


Figure 5-1. Time dependent changes in TNF-α mRNA expression in maxilloturbinates. Bars represent the group mean ± S.E.M. (n = 8/group). *Significantly different from air-exposed controls ($p \leq 0.05$).

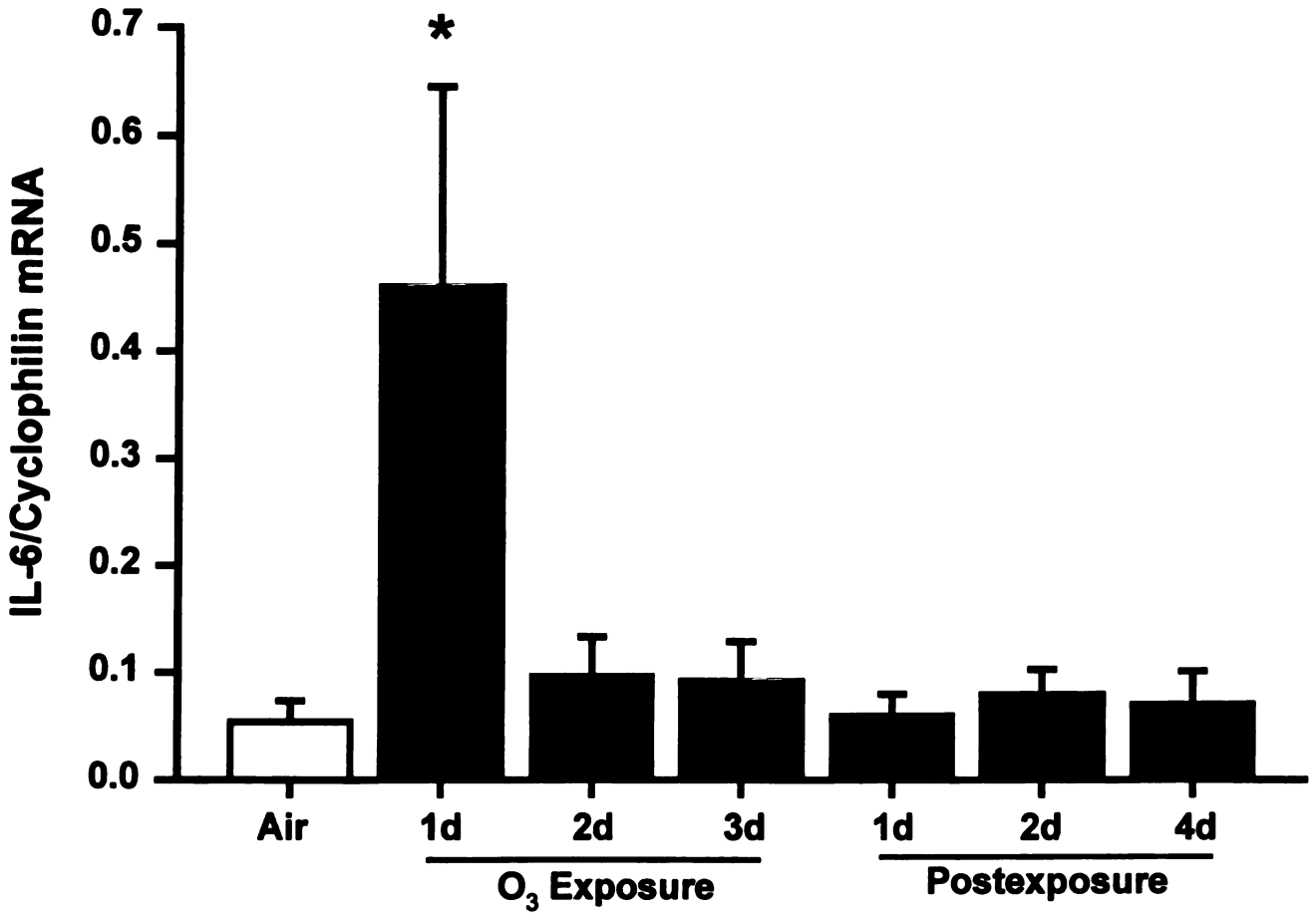


Figure 5-2. Time dependent changes in IL-6 mRNA expression in maxilloturbinates. Bars represent the group mean ± S.E.M. (n = 8/group). *Significantly greater than air-exposed controls ($p \leq 0.05$).

Discussion

In the present study, we demonstrated a distinctive induction of mRNAs for two pro-inflammatory cytokines, TNF-α and IL-6, in rat nasal airways after single and/or repeated exposure to ozone. There was a tendency of increases in nasal TNF-α mRNA levels in rats killed 2 h after exposure to ozone for 1 and 2 days (70% more than controls), although significant increases were observed only in rats killed 2 h after 3 days of exposure (110% more than controls). The level of IL-6 mRNA was elevated only in rats killed 2 h after 8 h (*i.e.*,1 day) of ozone exposure. Numerous studies have correlated increased cytokine mRNA levels with increased production of this cytokine (Elias *et al.*, 1990). Therefore, our results suggest that acute ozone induces a rapid local production of TNF-α and IL-6 in nasal tissues.

In the previous study (Chapter 2), we observed an ozone-induced transient neutrophilic influx in the NTE during the first 3 days of ozone exposure, which was resolved after the end of exposure (See Figure 2-5 in page 65). TNF-α and IL-6 mRNA levels increased at the same time of neutrophilic influx in the nasal tissues. Our observations suggest that TNF-α and IL-6 may be involved in the recruitment of neutrophils to the site of ozone-induced injury, or that these cytokines may be produced by infiltrating neutrophils. Increased levels of TNF-α and IL-6 mRNA were also concurrent with the initial increases of rMuc-5AC mRNA levels in the ozone-exposed nasal tissues as we observed in Chapter 2 (See Figure 2-11 in page 71). To determine the direct effects of these ozone-inducible cytokines on mucin gene expression in nasal

tissues, we conducted an *in vitro* exposure of explants of microdissected maxilloturbinates to soluble TNF-α or IL-6 in the absence of neutrophils or other soluble substances (See Study 2 in page 149).

Previous studies suggested that TNF-α and IL-6 are involved in airway repair or adaptive responses. TNF-α has been linked to the initiation of silica-induced tissue reparative adaptation, such as fibrosis in rat lungs (Piguet *et al.*, 1989, 1990). It has also been reported that administration of TNF-α can attenuate ozone-induced epithelial cell injury in rat NTE (Hotchkiss and Harkema, 1992). In the present study, we observed that TNF-α was elevated during the first 3 days of ozone exposure, which may be the most critical time for the molecular and cellular changes to develop MCM in the NTE. Therefore, TNF-α may be an important factor in ozone-induced MCM. Though the overexpression of IL-6 message was more brief than that of TNF-α after ozone exposure in our study, IL-6 was persistently expressed in pulmonary airways of rats exposed to chronic ozone (0.5 ppm, 56 days) (Bree *et al.*, 1996). In addition, results from a recent study indicated that inhibition of IL-6 by pretreatment with antibody exacerbated the acute ozone-induced neutrophilic inflammation and tissue injury in rat lungs (McKinney *et al.*, 1998). These previous findings suggest a possible role of IL-6, as an anti-inflammatory cytokine, in airway repair and adaptation after ozone exposure.

STUDY 2: EFFECTS OF SOLUBLE TNF-α AND IL-6 ON RAT MUC-5AC mRNA EXPRESSION

Materials and Methods

Animal, microdissection of maxilloturbinate and culture. Twelve male F344/N rats (Harlan Sprague-Dawley, Indianapolis, IN), 10 - 12 weeks of age, were used to determine effect of TNF-α on rMuc-5AC mRNA expression. Eighteen rats were used to determine the effect of IL-6 on rMuc-5AC mRNA expression. Rats were sacrificed and maxilloturbinates from nasal passages were removed immediately by microdissection as described in Chapter 2. Each microdissected maxilloturbinate was randomly assigned to one of 6 experimental groups in each study (n = 4/group for TNF-α study, n = 6/group for IL-6 study). The maxilloturbinates were placed on 0.4 μm-pore-size Transwell inserts (Costar, Pleasanton, CA) in 12-well plates (1 maxilloturbinate/well). The maxilloturbinates were placed in the center of the mesh insert with medial aspects of the turbinates facing the air phase (Figure 5-3). Medium was added to both the top (100 μl) and bottom (400 μl) compartments of the Transwell. The culture medium (supplemented Ham's F12), a modification of that used by Wu and coworkers (Wu *et al.*, 1985), contained 1 μM hydrocortisone, 5 μg/ml transferrin, 5 μg/ml insulin, 25 ng/ml EGF, bovine pituitary extract, 1 μM retinol, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The tissues were cultured (37°C, 5% CO_2) overnight.

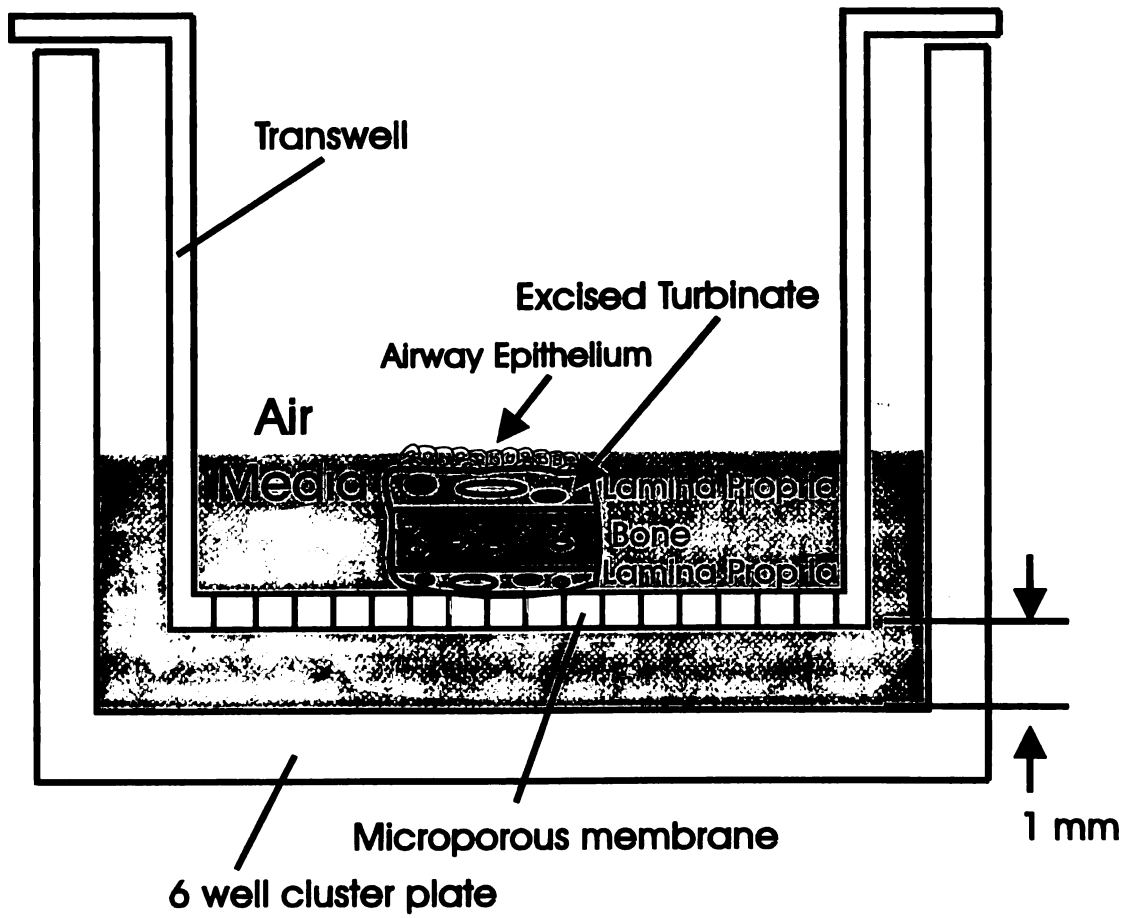


Figure 5-3. Culture of microdissected nasl tissue

In vitro exposure. Recombinant human TNF-α (rhTNF-α; R&D System, Minneapolis, MN) or recombinant human IL-6 (rhIL-6; Gibco BRL, Gaithersburg, MD) was dissolved in sterile phosphate-buffered saline containing 0.1 % bovine serum albumin (Sigma Chemical Co., St.Louis, MO) as a carrier. Maxilloturbinates in culture were exposed to either TNF-α or IL-6 by replacing the medium in the upper (80 μl) and lower (400 μl) chambers of the Transwell with fresh supplemented F12 containing 0 (control), 2, or 20 ng/ml of the respective cytokine. Maxilloturbinates cultured with TNF-α were removed and analyzed after 4 and 24 h of exposure. Maxilloturbinates cultured with IL-6 were removed and analyzed after 8 and 24 h of exposure. The concentration and the exposure duration were chosen based on previously published studies in which exogenous TNF-α or IL-6 was shown to effect various cellular and molecular changes including activation of cellular genes *in vitro* without cytotoxicity (Levine *et al.*, 1995, 1994; Roncero *et al.*, 1995; Ravid *et al.*, 1995; Rokita *et al.*, 1989; Putowski and Kotarski, 1995). Lactate dehydrogenase (LDH) level in the culture medium was measured as an indicator of tissue cytotoxicity caused by each cytokine. At the end of culture, the activity of LDH in the medium was determined spectrophotometrically from the disappearance of NADH using pyruvate as substrate. Total RNA was isolated from each tissue, and analyzed for rMuc-5AC mRNA expression using quantitative RT-PCR assay.

Preparation of internal standard for rMuc-5AC RT-PCR. We followed the simple and rapid general PCR-based method reported by Heuvel *et. al.* (Vanden Heuvel *et al.*, 1994) for generating rcRNA templates for use as internal standards in quantitative RT-PCR.

The basis for this method is amplification of genomic DNA using primers containing the T7 promoter, target (*i.e.*, rMUC-5AC), and spacer RNA (*i.e.*, human glutathion S-transferase; GST) primer sequences and polydeoxythymidylic acid ($poly(dT)_{18}$) (Figure 5-4). Genomic DNA was used to generate PCR products of an appropriate size to be resolved from the target RNA-PCR products. The T7 promoter and $poly(dT)_{18}$ are needed to produce a rcRNA with a polyadenylated tail following *in vitro* transcription of the PCR products. Using this procedure, the rcRNA molecules which contained rMuc-5AC mRNA forward and reverse primer sequences were able to be synthesized, and a RT-PCR product was produced and easily resolved from that generated from the rMuc-5AC mRNA. Construction of the rcRNA internal standards was accomplished using the following procedure. The forward rcRNA primer contained the T7 promoter, rMUC-5AC mRNA forward primer, and GST mRNA forward primer. The reverse rcRNA primer contained the GST mRNA reverse primer, rMuc-5AC mRNA reverse primer, and $poly(dT)_{18}$. The rcRNA primers were approximately 60 base pairs in length and synthesized by the Southwest Scientific (Albuquerque, NM). PCR reactions were conducted in a final volume of 50 ml containing PCR buffer, 3 mM $MgCl_2$, 0.2 mM each of deoxynucleotide triphosphate, 30 pmol each of rcRNA forward and reverse primers, 100 ng human genomic DNA, and 1.25 units Taq DNA polymerase. The reactions were assembled at 85°C, heated to 94°C for 3 min, and cycled 30 times through a 15-sec denaturing step at 94°C, a 30-sec annealing step at 55°C, and a 30-sec extension step at 72°C. Following the final cycle, a 10-min extension step at 72°C was included. The PCR products were diluted 1:100 in water, and 2 ml were reamplified using the

conditions stated above. PCR products from several second amplification reactions were pooled and purified (Magic-Prep DNA purification system; Promega, Madison, WI). The pooled PCR products were transcribed into RNA by the T7 promoter using the Riboprobe Gemini II *in vitro* transcription system (Promega, Madison, WI). The rcRNA was subsequently treated with RNase-free DNase to remove the DNA template, extracted sequentially with water-saturated phenol-chloroform (24:1) and chloroform-isoamyl alcohol (24:1), and precipitated with ethanol. The rcRNA pellet was washed with 70% ethanol, suspended in nuclease-free water, and quantitated by absorbance at 260 nm.

Quantitative RT-PCR. Quantitative RT-PCR was performed as described by Gilliland *et al.* (Gilliland *et al.*, 1990). For each RNA sample, 6 equal aliquots (100 ng total RNA) were prepared, and a dilution series (0 or 10^5 - 10^7 molecules/tube) of the internal standard RNA was spiked into the RNA aliquot in each tube. Reverse transcription of RNA was performed following the same procedure described in semiquantitative RT-PCR analysis of rMuc-5AC mRNA (Chapter 2). The PCR amplification of cDNA products were followed by 3-min incubation at 95°C, 33-cycling of the three-step temperature reactions (95°C 30 sec, 56°C 1 min, 72°C 1 min), and final extension step at 72°C for 10 min using same reagents described in Chapter 2. Aliquots (15 ml) of the PCR reaction were electrophoresed as described previously. Quantitation of the amount of rMuc-5AC mRNA was performed using the method by Gilliland *et al.* (1990). Briefly, the ratio of the densitometric volume of internal standard cDNA spot to that of rMuc-5AC cDNA spot was plotted against the amount of internal standard RNA (number of

molecules) added to each reaction. The internal standard RNA concentration at which the volume ratio was equal to one (*i.e.*, where the volume of rMuc-5AC cDNA matches that of the internal standard RNA) represented the amount of rMuc-5AC mRNA present in the initial RNA sample. A broad range of internal standard concentration (*i.e.*, 10^1 to 10^8 molecules/tube) was examined in order to estimate an approximate concentration of the rMuc-5AC mRNA in each tissue sample. In a second RT-PCR, a much narrower internal standard range (*i.e.*, 10^5 to 10^7) was examined for accurate quantitation of mucin message levels.

Statistical analyses. All data were expressed as the mean group value ± the SEM. The mucin mRNA data from individual cytokine study were analyzed by two-way ANOVA to determine the potential effects of TNF-a or IL-6 concentration and exposure duration on mucin mRNA expression. Student-Newman-Keuls Method was followed to determine the significant differences in IL-6-induced group mean values ($p \leq 0.05$). Because there was no effect of exposure duration (*i.e.*, 4 or 24 h) on TNF-α-induced rMuc-5AC mRNA expression, data from each concentration of TNF-α-exposed groups (*i.e.*, 0, 2 and 20 ng/ml) were pooled. The pooled data were then analyzed by the Analysis of Contrast to determine the significant differences in the mean rMuc-5AC mRNA levels among the combined experimental groups ($p \leq 0.05$). The data from LDH assay of each study were also analyzed by two-way ANOVA and following Student-Newman-Keuls Method to determine the potential cytotoxicity of individual cytokines in nasal tissues ($p \leq 0.05$).

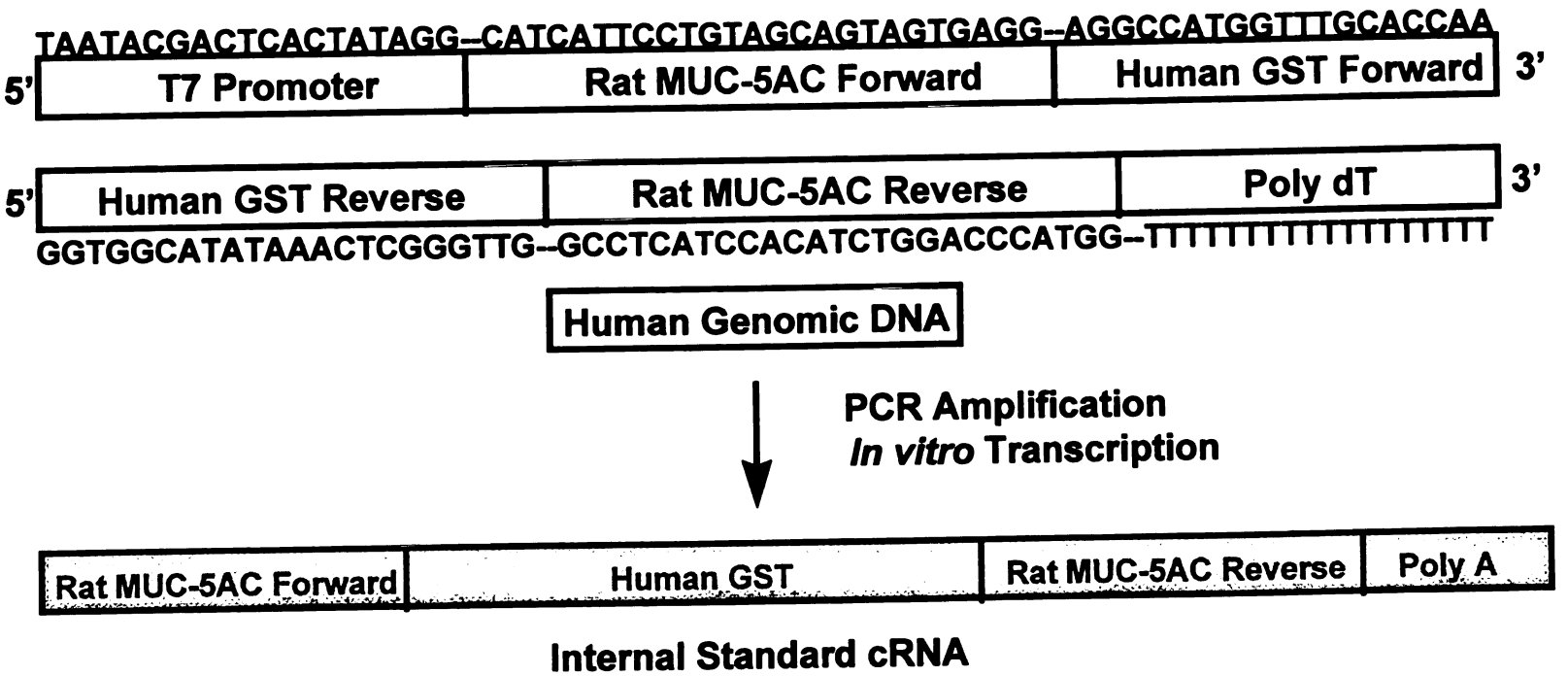


Figure 5-4. Design of a rat MUC-5AC internal standard for use in quantitative RT-PCR analysis to measure steady-state levels of rMuc-5AC mRNA. GST = glutathione-S-transferase; Forward = forward primer sequence; Reverse = reverse primer sequence.

Results

Effect of TNF-a on rMuc-5AC mRNA Expression. Figure 5-5 depicts digitized images of representative agarose gels containing rMuc-5AC and IS cDNA bands and standard plots used in the densitometric determination of rMuc-5AC mRNA levels in maxilloturbinates. The volume ratio of the internal standard cDNA band to rMuc-5AC cDNA band *versus* the amount of the internal standard added was a linear relationship (correlation coefficient; 98 - 100 %). A slight increase in rMuc-5AC mRNA level (2.5 to 4.1 X 10^6 molecules/mg RNA) was observed in cultured tissues receiving 2 ng/ml of TNF-α, but this was not significant, compared to the message level in control tissues (Figure 5-6). However, exposure of maxilloturbinates to 20 ng/ml of TNF-α resulted in a 3-fold increase (2.5 to 7 X 10^6 molecules/mg RNA) in the rMuc-5AC mRNA expression. No evidence of TNF-α-induced cytotoxicity was detected by LDH determination at both concentrations (Figure 5-7).

Effect of IL-6 on rMuc-5AC mRNA Expression. The steady-state level of rMuc-5AC mRNA in the cultured maxilloturbinates increased 2-fold (0.9 to 1.7 x 10^7 molecules/mg RNA) after 24 h exposure to 2 or 20 ng/ml of IL-6 (Figure 5-8). A linear relationship (correlation coefficient; 77 - 97 %) was observed in the ratio of the volume of the internal standard to rMuc-5AC mRNA *versus* the amount of the internal standard added. No evidence of IL-6-induced cytotoxicity was detected by LDH determination at both concentrations (Figure 5-9).

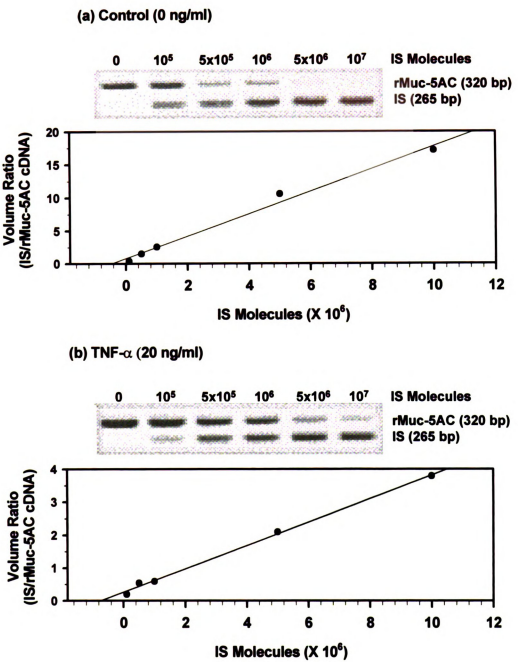


Figure 5-5. Digitized images of representative agarose gels and standard plots for rMuc-5AC mRNA quantitation using rMuc-5AC-specific internal standard (IS) rcRNA. As the amount of IS in the reaction increases, the volume ratio increases. The number of rMuc-5AC mRNA molecules is determined by the number of IS where the volume ratio equals 1.

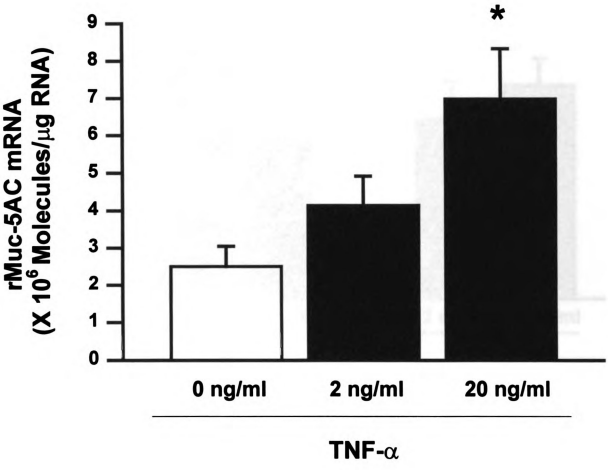


Figure 5-6. Effect of TNF-α on rMuc-5AC mRNA expression in explants of maxilloturbinates. Bars represent the group mean ± S.E.M. (n = 4/group). *Significantly different from the control ($p \leq 0.05$).

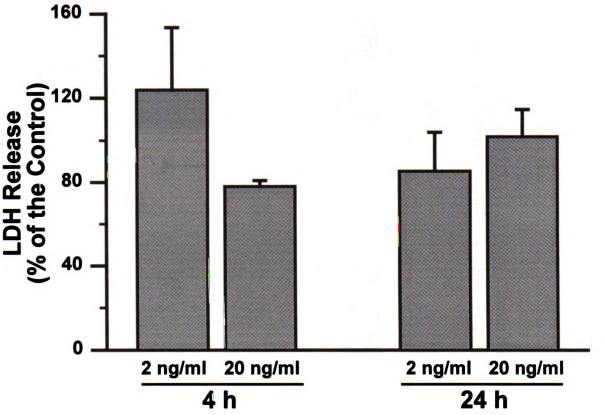


Figure 5-7. Cytotoxicity of TNF-α to maxilloturbinate explants indicated by % LDH release compared to time-matched, vehicle-exposed controls. Bars represent the group mean ± SEM (n = 4/group).

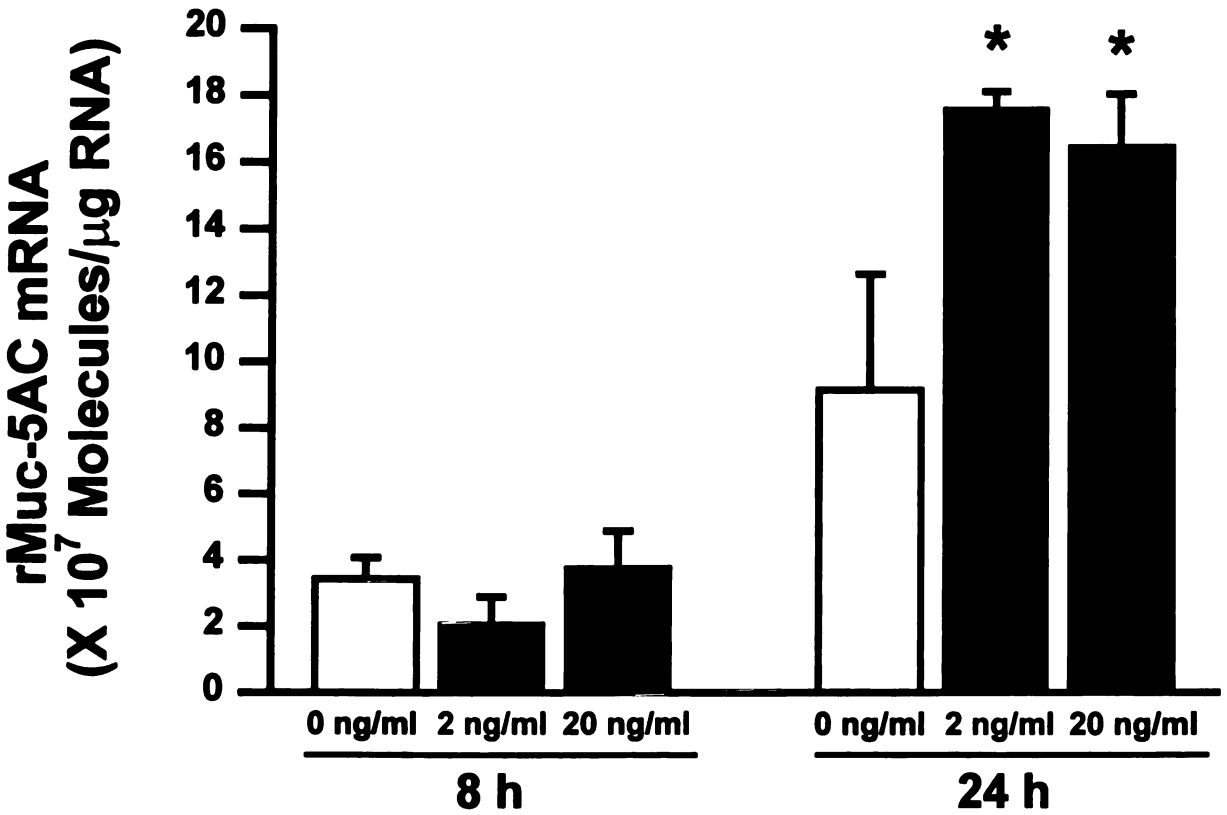


Figure 5-8. Effect of IL-6 on rMuc-5AC mRNA expression in explants of maxilloturbinates. Bars represent the group mean ± SEM (n = 6/group). *Significantly different from the controls ($p \leq 0.05$).

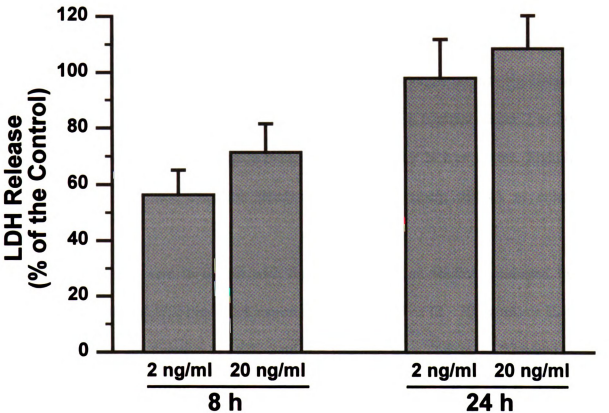


Figure 5-9. Cytotoxicity of IL-6 to maxilloturbinate explants indicated by % LDH release compared to time-matched, vehicle-exposed controls. Bars represent the group mean $\pm$ SEM (n = 6/group).

Discussion

The results of the present study demonstrate that the ozone-inducible pro-inflammatory cytokines, TNF-α and IL-6, can directly induce increased rMuc-5AC mRNA levels in explants of microdissected maxilloturbinates. Exposure of nasal tissues to 20 ng/ml of TNF-α induced a 3-fold increase in rMuc-5AC mRNA, compared to vehicle-exposed controls. At 2 ng/ml of TNF-α, there was a slight but not significant increase in mucin message levels, compared to the controls. In contrast, either 2 or 20 ng/ml of IL-6 induced 2-fold increases in rMuc-5AC mRNA after 24 h exposure. Eight h exposure to IL-6 did not alter the steady-state level of mucin mRNA at either concentration.

Our observations were in accord with the results of recent studies conducted by Levine *et al.* (1994 and 1995) in which exposure to either TNF-α (2 - 20 ng/ml) or IL-6 (20 ng/ml) induced upregulation of MUC-2 mRNA (0.5 - 24 h by TNF-α, 8 - 72 h by IL-6) in cultured human airway epithelial cells. In those studies, TNF-α and IL-6 also induced concurrent mucin hypersecretion in cultured cells (TNF-α; 4 - 72 h, IL-6; 8 - 72 h) or bronchial organ explants (TNF-α; 1 - 24 h, IL-6; 4 - 48 h) at the same concentrations. In addition to TNF-α and IL-6, IL-4 and IL-5 have been implicated in mucin gene upregulation, mucin hypersecretion and/or MCM in distal airways of rodents (Rankin *et al.*, 1996; Lee *et al.*, 1997; McBride *et al.*, 1994). Though IL-1b is a strong mucin secretagogue in airway epithelial cells (Jarry *et al.*, 1996), little is known about its role in mucin gene expression. The results of these recent studies indicate that certain

cytokines (*i.e.*, TNF-α , IL-4 and IL-6) can have multiple effects on airway mucous cells - inducing mucin gene expression, mucous hypersecretion, or overproduction. Therefore, it is strongly postulated that TNF-α and/or IL-6 may play roles, not only in rMuc-5AC mRNA expression, but in mucin secretion or MCM in the nasal tissues of rats exposed to ozone.

In the present study, *in vitro* tissue culture was employed to assess the direct effects of individual cytokines on mucin gene expression. By using this culture system, we eliminated possible effects of neutrophils or neutrophil-mediated inflammatory responses on mucin mRNA regulation in nasal tissues. The results of the present study suggested that local mucosal production of the cytokines, TNF-α and IL-6, may have had direct effects on the ozone-induced mucin gene upregulation in nasal tissues. Further studies are required to determine if these cytokines are expressed by nasal epithelial cells or other mucosal cells in ozone-exposed nasal airway.

CHAPTER 6

Summary and Conclusions

Epithelial alterations in the nasal airways of rats exposed to acute or chronic ozone have been well characterized in the previous studies from our laboratory. Acute exposure of rats to high ambient concentrations of ozone (3 - 7 days, $\geq$ 0.5 ppm) induces MCM and epithelial hyperplasia in the NTE lining the lateral meatus of proximal nasal airways in rats (Harkema *et al.*, 1989, 1994; Hotchkiss *et al.*, 1989). A transient neutrophilic inflammation precedes the development of epithelial hyperplasia and MCM (Harkema *et al.*, 1989; Hotchkiss *et al.*, 1989, 1997). These previous studies, however, were not designed to investigate how the epithelial alterations (*i.e.*, neutrophilic inflammation, epithelial hyperplasia, MCM) relate to each other. In addition, the effects of ozone exposure on airway mucin-specific gene expression and its relationship to the ozone-induced MCM had not been previously investigated. To further investigate the pathogenesis of ozone-induced MCM, we designed the present research to test the guiding hypothesis that neutrophilic inflammation plays a key role in the development of MCM in the NTE.

To understand how and when ozone induces nasal cell injury and the reparative and adaptive changes (*i.e.*, epithelial proliferation and MCM) that occur in the NTE of ozone-exposed rats, we first determined the time-dependent relationships of neutrophilic inflammation with epithelial responses including airway mucin-specific gene (rMuc-5AC) expression and epithelial proliferation (*i.e.*, NTE cell BrdU-labeling index and numeric density) which occurred early after the start of exposure and during the development of MCM. We also investigated the temporal relationships of ozone-induced mucin mRNA upregulation with epithelial proliferation and MCM. The results from our studies demonstrated that acute ozone exposure (0.5 ppm, 8 h) induced an increase in the

steady-state levels of rMuc-5AC mRNA in nasal tissues within hours after the start of exposure. This ozone-induced upregulation of the airway mucin gene preceded the phenotypic expression of MCM by three days. The upregulation of this mucin gene was initiated concurrently with neutrophilic inflammation in the NTE, but persisted with epithelial hyperplasia and MCM even after the initial neutrophilic inflammation was resolved two days later. Although temporal correlations of those molecular and cellular responses in the present study did not prove causality, these findings suggested that the early ozone-induced increases in mucin mRNA are associated with the neutrophilic inflammation and epithelial cell proliferation. In addition, ozone-induced MCM may be dependent on these important pre-metaplastic responses (*i.e.*, mucin mRNA upregulation, neutrophilic inflammation and epithelial proliferation).

Based on these initial findings, we further investigated the role of infiltrating neutrophils in the ozone-induced epithelial alterations (*i.e.*, hyperplasia and MCM) and mucin gene upregulation in the NTE. For this purpose, we depleted rats of their circulating neutrophils using an anti-rat neutrophil antiserum prior to the repeated, acute, ozone exposures. The results from this study indicated that ozone-induced MCM is, at least in part, neutrophil-dependent (*i.e.*, ~70 % decrease of ozone-induced MCM in antiserum-treated animals, compared to control serum-treated animals). In contrast, we found that the induced epithelial proliferation (*i.e.*, epithelial DNA synthesis and hyperplasia) and the increase in mucin mRNA levels were independent of the ozone-induced neutrophilic influx in the NTE. Therefore, it was concluded that ozone alone is sufficient to induce mucin gene upregulation and epithelial proliferation in the NTE

before the onset of MCM. However, for full phenotypic expression of MCM, neutrophil-mediated molecular and cellular events are needed.

Another study was then designed to support the neutrophil-dependency of the ozone-induced MCM. We hypothesized that pre-existing neutrophilic inflammation in the NTE (*i.e.*, rhinitis) augments the ozone-induced MCM. For this purpose, neutrophilic rhinitis was induced by exposing rats to a strong pro-inflammagen, endotoxin (100 μg/day, intranasal instillation), 6 h prior to daily ozone exposure (0.5 ppm, 8 h/day for 3 days). Pretreatment of endotoxin enhanced the severity of the ozone-induced MCM by 2 fold, while it did not affect the severity of either NTE cell proliferation or mucin mRNA upregulation induced by ozone. Although endotoxin alone induced neutrophilic inflammation and mucin mRNA upregulation, it did not induce MCM in the NTE. The results of this study suggested that the augmentation of ozone-induced MCM could be mediated by endotoxin-induced inflammatory responses. However, we cannot rule out the direct contribution of endotoxin to the pathogenesis of MCM in the NTE of ozone-exposed nasal airways.

Our *in vivo* findings suggested that mucin mRNA levels in nasal tissues do not always match with or predict the histologic appearance of the mucin glycoproteins. However, significant increases of the mucin mRNA levels were consistently detected prior to the onset of the ozone-induced MCM in the NTE. A few previous studies have also indicated that other airway irritants (e.g., sulfur dioxide, acrolein) also induce upregulation of mucin mRNA concurrently with or prior to the development of MCM in distal airway epithelium of rats (Jany *et al.*, 1991; Borchers and Leikauf, 1997).

However, underlying mechanisms of the elevated expression of airway mucin gene have not been fully investigated. Recently, several cytokines (e.g., TNF-α, IL-4, IL-5, IL-6) have been reported to play important roles in mucin mRNA upregulation in association with elevated mucous production in or secretion from airway epithelial cells (Levine *et al.*, 1994 and 1995; Rankin *et al.*, 1996; Temann *et al.*, 1997; Lee *et al.*, 1997). Among them, TNF-α and IL-6 are known to be produced by airway epithelial cells after ozone exposure in the absence of inflammatory cells (Devlin *et al.*, 1994; Beck *et al.*, 1994). In our rat model of ozone exposure, we observed that expression of mRNAs for these two pro-inflammatory cytokines, TNF-α and IL-6, was markedly increased in nasal tissues as early as 10 h after the start of ozone exposure. Induction of these cytokine genes coincided with increases in mucin mRNA levels in nasal tissues. Therefore, we conducted *in vitro* studies to investigate the putative role of those ozone-inducible cytokines (*i.e.*, TNF-α, IL-6) in mucin gene expression in nasal tissues. When soluble forms of either TNF-α or IL-6 (2 or 20 ng/ml, 4 - 24 h) were applied to the explants of microdissected maxilloturbinates, the steady-state level of rMuc-5AC mRNA was elevated by 2 - 3 fold. These results suggested that local mucosal production of TNF-α and IL-6 may directly contribute to the ozone-induced mucin mRNA upregulation in nasal tissues.

Major findings of our studies are summarized in a diagram in Figure 6-1. Ozone directly induces early overexpression of mucin mRNA and epithelial cell proliferation in the absence of neutrophilic inflammation. These neutrophil-independent epithelial events contribute to some extent to the development of the ozone-induced MCM. However,

neutrophilic inflammation plays a key role in the full phenotypic development of MCM, probably by accelerating mucin apoprotein translation or mucin glycosylation (e.g., increases in N-acetylgalactosaminetransferase activity or its expression, mucin transport through RER and Golgi), or by stabilizing stored mucin in granules or inhibiting mucin exocytosis.

How these inflammatory cells mediate this epithelial transformation is yet to be determined. Future studies must be designed to determine if soluble substances derived from neutrophils (e.g., proteases, cytokines, peptide growth factors) are responsible for the metaplastic transformation of NTE from a nonsecretory epithelium to a secretory epithelium containing numerous mucous cells. In addition, further research is needed to understand what specific epithelial cells (e.g., basal, cuboidal) are involved in these metaplastic processes. What are the progenitor cells for the newly appeared mucous cells ? Answers to these questions will not only add to our understanding of how ozone causes MCM in the NTE of rats, but will also contribute to a greater understanding of the cellular and molecular mechanisms involved in this basic metaplastic process which is a key pathologic feature in most chronic airway diseases (e.g., asthma, chronic bronchitis, allergic rhinitis, and cystic fibrosis).

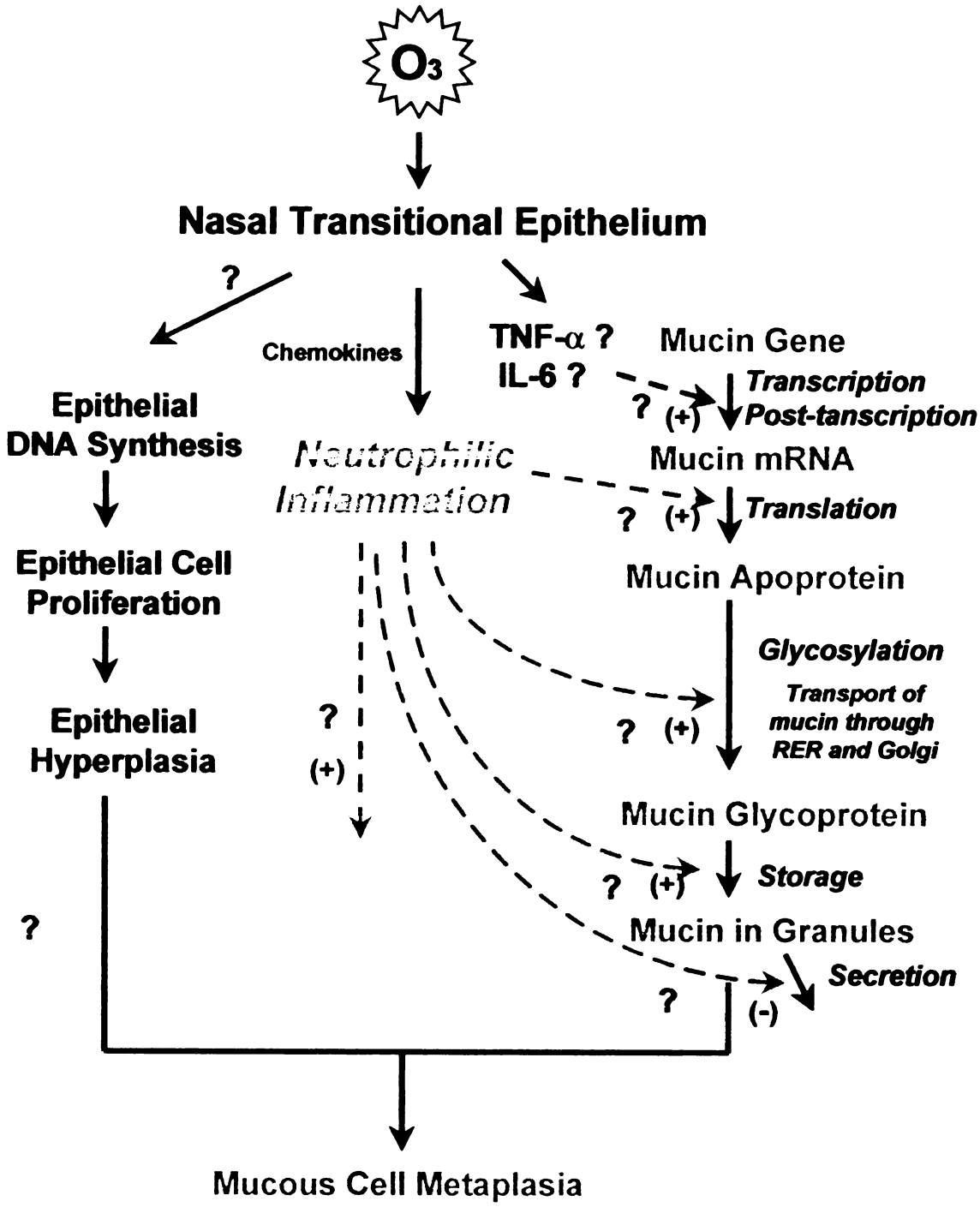


Figure 6-1. Summarized diagram of results

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