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Mechanisms of Response to Ozone Exposure: The Role of Mast Cells in Mice

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Includes the Commentary of the Institute's Health Review Committee

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HEI Statement

Synopsis of Research Report Number 85

Mechanisms of Response to Ozone Exposure: The Role of Mast Cells in Mice

BACKGROUND

Ozone is a highly reactive gas and a major component of photochemical oxidant air pollution. Short-term exposure to ozone can cause a transient migration of inflammatory cells to the airways. Less is known about the effects of prolonged or repeated exposures. There is concern, however, that such exposures may cause chronic inflammation that could permanently damage the small airways because inflammatory cells release substances that injure the epithelial cells lining airway surfaces. Mast cells (a type of inflammatory cell) are thought to play an important role in lung inflammation because they reside in lung tissues and produce substances that stimulate the migration and growth of other inflammatory cells, which in turn produce substances that injure epithelial cells. HEI supported a study by Dr. Steven Kleeberger and colleagues of Johns Hopkins University that was designed to improve our understanding of the mast cell's contribution to lung inflammation and epithelial cell injury caused by exposure to ozone.

APPROACH

Kleeberger and colleagues compared ozone-induced inflammation, epithelial cell injury, and epithelial cell proliferation (a marker of cell injury) in three types of mice: mice with a normal content of mast cells, mutant mice without mast cells, and mutant mice whose mast cells were repleted by a bone marrow transplant from normal mice. (Bone marrow is the source of several cell types, including mast cell precursors that migrate to tissues and become mature mast cells.) Each group of mice was exposed to clean air or to 0.26 parts per million (ppm) ozone for 1, 3, 14, 30, or 90 days for 8 hours per day, 5 days a week (for exposures longer than 3 days). To determine whether the effects of prolonged ozone exposure reversed, the investigators exposed a separate set of mice from each group to ozone for 90 days and allowed them to breathe clean air for 35 days before they measured inflammation, cell injury, and cell proliferation.

RESULTS AND INTERPRETATIONS

Exposure to ozone induced more inflammation, epithelial cell injury, and epithelial cell proliferation in the lungs of mice with normal levels of mast cells than in mast cell–deficient mice. All changes, with the notable exception of epithelial cell proliferation, returned to control levels in normal mice after a 35-day recovery in clean air. In general, mast cell–deficient mice whose mast cell levels were repleted responded to ozone like normal mice. These results support the investigators' hypothesis that mast cells contribute to ozone-induced inflammation and epithelial cell injury. However, because bone marrow transplantation replenishes more than mast cells, the contribution of other cell types to the ozone-induced changes in these mice cannot be ruled out. The results of this study are an important contribution to understanding the mechanisms of lung injury that may occur in people exposed to ozone. Furthermore, the persistence of increased epithelial cell proliferation after ozone exposure stopped suggests the possibility of permanent, ongoing structural changes. These findings could be especially relevant for individuals with asthma, who have more mast cells in their lungs than other healthy people.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1995 to 1997. This study was conducted by Drs. Steven R. Kleeberger, Malinda Longphre, and Clarke G. Tankersley at the Division of Physiology, School of Public Health, Johns Hopkins University. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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Mechanisms of Response to Ozone Exposure: The Role of Mast Cells in Mice

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ABSTRACT

Acute and subacute exposure to ozone (O_3)* induces lung inflammation and hyperpermeability and causes epithelial injury of both upper (nasal) and lower airways. Mast cells are important regulatory cells in mice for each of these effects. Subacute and chronic O_3 exposures cause epithelial injury and inflammation in terminal bronchioles and proximal alveoli. Little is known, however, about the mechanisms of injury. Because inflammatory processes may be linked to the pathogenesis of many airway diseases, it is critical to understand the underlying mechanisms that initiate and propagate these processes. We tested the hypothesis that mast cells mediate airway injury induced by chronic O_3 exposure by comparing regional airway inflammation and epithelial injury as well as ventilatory responses in genetically mast cell–deficient mice ($WBB6F_1$-Kit^W/Kit^{W-v} [Kit^W/Kit^{W-v}]) with those in (1) normal, mast cell–sufficient, congenic littermates ($WBB6F_1$-+/+ [+/+]) and those in (2) Kit^W/Kit^{W-v} mice that were repleted with mast cells by bone marrow transplantation (BMT) from +/+ donors (Kit^W/Kit^{W-v}-BMT). Thus, three (different) groups of mice were used.

The following experimental protocol was utilized to test this hypothesis. Animals from each treatment group (n = 4–6/group) were exposed to 0.26 parts per million (ppm) O_3 8 hours/day and 5 days/week for durations of 1, 3, 14, 30, and 90 days. Between 8-hour exposures, mice were exposed continuously to 0.06 ppm O_3. Age-matched mice were simultaneously exposed to filtered air (0.0 ppm O_3) to serve as O_3 controls. To evaluate reversibility of exposure-induced lesions, a set of mice from each genotypic group was exposed to air or O_3 for 90 days and then placed in HEPA-filtered air for 35 days. After each period of exposure and after 35-day recovery, the nasal cavity and lungs of O_3- and air-exposed mice from each group were evaluated for regional inflammation and permeability, epithelial proliferation, and ventilation pattern.

Estimates of airway inflammation and hyperpermeability were obtained by analysis of cell differentials and total protein concentrations, respectively, in fluids obtained through use of bronchoalveolar lavage (BAL). Ozone exposure caused significantly greater increases in lung macrophages, epithelial cells, and polymorphonuclear leukocytes (PMNs) in mast cell–sufficient +/+ and Kit^W/Kit^{W-v}-BMT mice than in mast cell–deficient Kit^W/Kit^{W-v} mice. Comparable ozone exposure also elicited increases in lung lymphocytes and in total protein, but there were no significant differences in these two genotypic groups. Cell and total-protein responses in BAL fluid returned to control levels (that is, air exposure only) in all three groups of mice after a 35-day recovery period.

The effects of O_3 exposure on cell proliferation in the nose and lung were evaluated in the genotypic groups by counting the number of cells that incorporated bromodeoxyuridine (BrdU, a thymidine analog) into DNA. In the centriacinar region of the lung, DNA synthesis was increased significantly in O_3-exposed +/+ and Kit^W/Kit^{W-v}-BMT mice, but not in Kit^W/Kit^{W-v} mice, compared with DNA synthesis in air controls. Epithelial proliferation remained significantly elevated or even increased in +/+ and Kit^W/Kit^{W-v}-BMT mice after O_3 exposure. Nasal responses to O_3 were also evaluated in these three genotypic groups of mice, and there were slight, although statistically significant, O_3-exposure effects on the transitional epithelium. However, there were no differences among the groups up to an exposure of 90 days in duration. After a 35-day recovery period, epithelial cell proliferation in +/+ and Kit^W/Kit^{W-v}-BMT mice was greater than that in Kit^W/Kit^{W-v} mice.

There were no significant exposure, genotype, or duration effects on baseline ventilation or responses to hypercapnic hypoxia in the three groups of mice exposed to air or O_3. However, significant interaction among the three variables

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report Number 85, which also includes a Commentary by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Steven Kleeberger, Department of Physiology, Rm. 7006, School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205.

Although this document was produced with partial funding by the United States Environmental Protection Agency (EPA) under Assistance Award R824835 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review. Therefore, it may not necessarily reflect the views of the Agency and no official endorsement by the EPA should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

indicated that there were genotype-specific effects of O_3 on ventilation after a 1-day exposure; these effects were not seen after longer exposures.

Results of these experiments are consistent with the hypothesis that mast cells significantly affect the pathogenesis of bronchiolar epithelial injury and proliferation induced by subchronic or chronic exposure to an environmentally relevant concentration of O_3 (that is, 0.26 ppm). Inasmuch as mast cell densities are increased in the airways of persons with allergic asthma, this effect of ozone exposure may have important implications in understanding why persons with asthma are more susceptible to inhaled pollutants.

INTRODUCTION

The oxidant potential of O_3 is well recognized, and this highly reactive pollutant is nearly ubiquitous in urban environments of the United States and other industrialized countries. In healthy human subjects, 2 to 6 hours of exposure at levels of 0.08 to 0.40 ppm O_3 causes a detectable decrease in pulmonary function (McDonnell et al. 1983; Aris et al. 1991; Weinmann et al. 1995), elicits the release of bronchoactive chemical mediators (Koren et al. 1989; Devlin et al. 1991), and induces both airway inflammation (Koren et al. 1989; Devlin et al. 1991; Schelegle et al. 1991; Aris et al. 1993) and rapid shallow breathing (Weinmann et al. 1995). Interestingly, there has been no demonstrated link between the intensity of the inflammatory response to O_3 and the O_3-induced effect on pulmonary function (Schelegle et al. 1991; Balmes et al. 1996). Epidemiologic studies suggest that short-term O_3 exposure increases bronchial reactivity, reduces lung function, and is a general risk factor for childhood and adult respiratory disorders (Kinney et al. 1989; Spektor et al. 1989; White et al. 1994; Romieu et al. 1995; Stieb et al. 1996).

Although these studies have provided insight into the potential mechanisms of acute O_3-induced pulmonary injury, it remains unclear whether such acute responses are predictive of the airway effects of subchronic or chronic O_3 exposures. Recent investigations have used animal models to examine the effects of simulated urban chronic intermittent O_3 exposure on airways (Harkema et al. 1987; Tepper et al. 1989; Chang et al. 1991, 1992; Last et al. 1993; Ito et al. 1994; Pinkerton et al. 1995). The animal studies have indicated that the most susceptible tissues in the upper (nasal) airways are the cuboidal and transitional epithelium, whereas the susceptible tissues of the lower airways are the conducting and respiratory epithelium and the alveolar epithelium. The effects of O_3 on nasal airways almost certainly compromise upper respiratory defense mechanisms, and they may also compromise the scrubbing efficiency of the same airways (Gerrity et al. 1988), thereby influencing the susceptibility of lung tissues. Although these studies provide useful insight into the time course and reversibility of chronic O_3-exposure effects, questions remain regarding the mechanisms that initiate and propagate the pathogenesis of airway injury induced by chronic O_3 exposure.

Several types of cells in the respiratory tract, including macrophages, epithelial cells, neutrophils, and neurons, are likely to be involved in responses to environmental stresses. However, there is increasing evidence that mast cells may be key to the stress response. Mast cells are found in large numbers in mammalian lungs and are closely associated with both pulmonary endothelium and nerve termini such as C-fibers (Bienenstock et al. 1989). When stimulated, mast cells may contribute significantly to bronchoconstriction, airway hyperreactivity, and inflammation. Proinflammatory mast cells have been implicated in a large number of disease processes including sarcoidosis, chronic fibrosing alveolitis, asthma, and allergic rhinitis (for review, see Kaliner and Metcalfe 1993). When stimulated, these cells release numerous mediators (such as tumor necrosis factor–alpha [TNF-α]) that were either preformed or de novo synthesized and that are critical to inflammatory cell recruitment and other events associated with tissue injury (Galli 1990; Gordon et al. 1990; Gordon and Galli 1990). Mast cells also contribute to normal tissue repair, and they have been associated with the early overproduction of basement-membrane components observed in a variety of fibrotic conditions (Thompson et al. 1991; Ramos et al. 1992).

Results from a number of studies suggest a role for mast cells in environmentally induced pulmonary disease, including observations in some animals exposed to O_3 that both mast cell degranulation (Dixon et al. 1965; Shields and Gold 1987; Kleeberger et al. 1989) and migration occur in the lungs. In healthy human subjects, acute O_3 exposure elicits the release of mast cell–specific tryptase into spaces of the upper and lower airways that can be sampled with BAL (Smith et al. 1993). Studies that have compared O_3-induced airway inflammatory responses in normal subjects and persons with asthma indicate that such responses are more severe in persons with asthma. This group of persons has preexisting allergic inflammation of the airways, a condition in which mast cells play a central role (Basha et al. 1994; Scannell et al. 1996). Although each of these studies implicates the mast cell in acute responses to O_3 exposure, the potential relation between these processes and subsequent physiologic responses or inflammatory

events that occur after subchronic and chronic O_3 exposure has not been established.

Investigations of the role of mast cells in pathologic and physiologic responses to specific and nonspecific agonists have been greatly enhanced by the development of genetically mast cell–deficient and mast cell–sufficient mice. Compared with normal, congenic WBB6F$_1$-+/+ mice (+/+), the mast cell–deficient WBB6F$_1$-Kit^W/Kit^{W-v} mouse (Kit^W/Kit^{W-v}) (formerly designated as WBB6F$_1$-W/W^v) is sterile, anemic, and lacks melanocytes in the skin. In addition, it has been characterized by a number of investigators as having less than 1% of normal mast cell densities in peripheral tissues and no mast cells present in the respiratory tract. However, the Kit^W/Kit^{W-v} mouse has normal levels of basophils and other granulocytes, T cells, and B cells (Galli and Kitamura 1987; Galli et al. 1992, 1994; Longphre et al. 1996b). Other studies with Kit^W/Kit^{W-v} mice have demonstrated that inflammatory responses to exogenous stimuli that are not mediated by mast cells are intact (Galli and Hammel 1984). The mast cell deficiency and associated abnormalities in Kit^W/Kit^{W-v} mice are due to a mutation at the *Kit* locus on chromosome 5, which results in a defect in the lineage of the receptor for stem cell factor that is expressed on the surface of hematopoietic progenitor cells, mast cells, melanocytes, and germ cells (Galli et al. 1994). The anemia and mast cell deficiency present in Kit^W/Kit^{W-v} mice can be repaired by adoptive transfer of bone marrow cells from +/+ donors, a procedure that has been described elsewhere (Nakano et al. 1985; Galli and Kitamura 1987; Galli et al. 1992; Kleeberger et al. 1993b). This model system of mast cell–sufficient and mast cell–deficient mice has been used to evaluate the role of mast cells in a number of immunologic and physiologic processes, including immediate hypersensitivity reactions, host defense, bleomycin-induced pulmonary fibrosis, sepsis, and silica-induced inflammation (Galli 1990; Suzuki et al. 1993; Echtenacher et al. 1996).

SPECIFIC AIM

The overall objective of this project was to test the hypothesis that mast cells contribute significantly to the initiation and propagation of inflammation, epithelial injury, and altered ventilatory function induced by subchronic or chronic O_3 exposure. The effects of acute and subacute exposures were also evaluated. A relatively complex study design was utilized to accomplish our objectives. Three independent variables (factors) were considered: genotype, exposure (O_3 or filtered air), and time. The dependent variables were indicators of inflammatory response (cells and total protein as obtained by BAL), evidence of epithelial injury (epithelial cell proliferation in upper and lower airways), and measures of ventilatory function (frequency and tidal-volume responses to room air and to challenge of exogenous hypercapnic hypoxia). Each of the dependent variables was analyzed as a function of the three independent variables.

METHODS

GENERAL METHODS

Male mast cell–deficient (Kit^W/Kit^{W-v}) and mast cell–sufficient (+/+) congenic mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were 6 to 8 weeks old and 20 to 25 grams of body weight at time of acquisition. Mice were housed in microisolation cages in an antigen- and virus-free room. Water and mouse chow (Agway Pro-Lab RMH 1000) were provided ad libitum. Sentinel mice were examined periodically (by titers and necropsy) by the Johns Hopkins University Department of Comparative Medicine to ensure that all animals remained free of infection. The mice were handled in accordance with the standards established by the U.S. Animal Welfare Acts as set forth in NIH guidelines and the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee.

BONE MARROW TRANSPLANTATION

Bone marrow transplantation (BMT) was performed to replete mast cell–deficient Kit^W/Kit^{W-v} mice with mast cells as described previously (Kleeberger et al. 1993b; Longphre et al. 1996) and as illustrated in Figure 1. Mast cell–sufficient, +/+ mice were killed by cervical dislocation, and their femurs were isolated. Bone marrow was obtained by flushing sterile saline through the bones into a sterile glass tube and suspending the cells in saline. The resultant marrow-cell suspension (1.5 mL, approximately 2.5×10^7 cells) was injected intraperitoneally into each Kit^W/Kit^{W-v} mouse. Age-matched +/+ and Kit^W/Kit^{W-v} mice were sham transplanted intraperitoneally with sterile saline (vehicle) as a control for the BMT procedure. All mice were housed for an additional 16 weeks before experimentation because full mast-cell reconstitution in airways has been shown to require at least 15 weeks (Kleeberger et al. 1993b).

To determine the success of mast cell repletion, the trachea and lungs were removed en bloc from separate groups of 25-week-old +/+, Kit^W/Kit^{W-v}, and Kit^W/Kit^{W-v}-BMT mice and were fixed in Carnoy's fixative (60:30:10, ethyl alcohol–chloroform–glacial acetic acid) at a constant pressure of 25 cm H_2O for 2 hours. The tissues were embedded in paraffin, sectioned (5 μm), and stained with

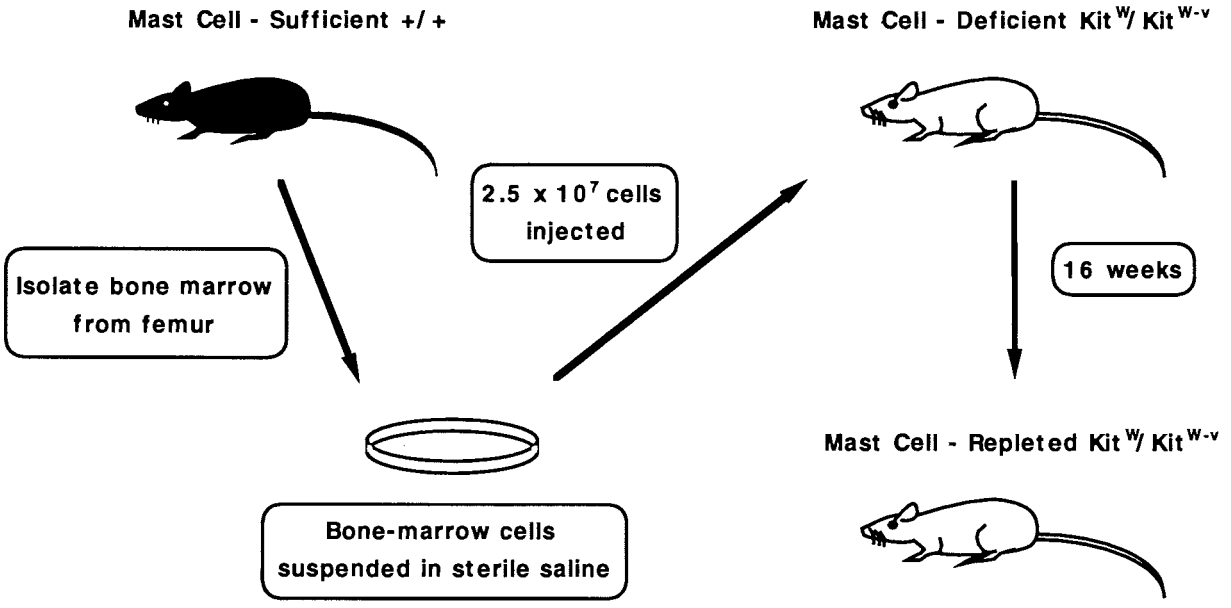

Figure 1. Bone marrow transplantation from +/+ mice to Kit^{W}/Kit^{W-v} mice. This schematic representation shows the procedures required to transplant bone marrow cells from mast cell–sufficient mice to mast cell–deficient mice.

toluidine blue. Sections were examined at a magnification of 400× for mast cells within or in juxtaposition to the tracheobronchiolar epithelium (one cell layer beneath basement membrane). Airway-lumen diameter of the trachea and mainstem bronchi were approximately 1.2 μm and 1.0 μm, respectively. These tissues were chosen for verification of mast cell repletion because previous studies have indicated that these tissues are normally relatively densely populated with mast cells and are good indicators of mast cell repletion throughout the lungs (Kleeberger et al. 1993b; Longphre et al. 1996b). All slides were coded, and mast cells were counted without knowledge of the treatment group. Sections were imaged as contour tracings on a digitizing tablet with use of camera lucida. The area examined for mast cells was calculated from the contour tracing with use of a Sigma Scan (Jandel Scientific, Corte Madera, CA). Longitudinal sections along the entire trachea or bronchus were examined for mast cells. The data for each group were expressed as the mean number of mast cells per μm^2 ± standard error of the mean (SEM). The trachea and mainstem bronchi were easily identified in the sagittal plane and were therefore the focus of study.

To determine the effect of BMT on blood-cell counts and hemoglobin and hematocrit, blood samples were taken from another group of 25-week-old +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v} -BMT mice by retroorbital bleeding. Samples were analyzed with a Cell-dyne 3500 Hematology Analyzer (Abbott Diagnostics, Abbott Park, IL).

OZONE EXPOSURES

All O_3 exposures were performed in the Inhalation Facilities of the Johns Hopkins University School of Hygiene and Public Health. During each exposure, mice were placed individually in stainless-steel wire cages with free access to food and water. The cages were set inside 700-L laminar-flow inhalation chambers that provided a charcoal- and HEPA-filtered air supply. Chamber air was renewed at the rate of approximately 20 changes per hour, with 50% to 65% relative humidity and an ambient temperature of 20° to 25°C. Ozone was generated by directing dried, filtered air through an ultraviolet-light O_3 generator (Orec Corp., Phoenix, AZ) located upstream of the exposure chamber. The O_3-air mixture was metered into the inlet air stream with the use of computer-operated, stainless-steel, mass-flow controllers. Simultaneous exposures to filtered air (0.0 ppm O_3) were done in a separate chamber for age- and treatment-matched groups of mice that served as O_3 controls. Intermittent O_3 exposures were performed automatically using a control program and microcomputer that were interfaced with the O_3-generating system. During each exposure, O_3 concentrations were monitored every 2 minutes at different heights in the chamber with the use of an O_3 ultraviolet photometer (Dasibi model 1003-AH, Dasibi Environmental Corp., Glendale, CA). Concentrations were recorded on a strip chart recorder. The cumulative means and standard errors of the O_3 concentration, as well as maximum and minimum exposure levels,

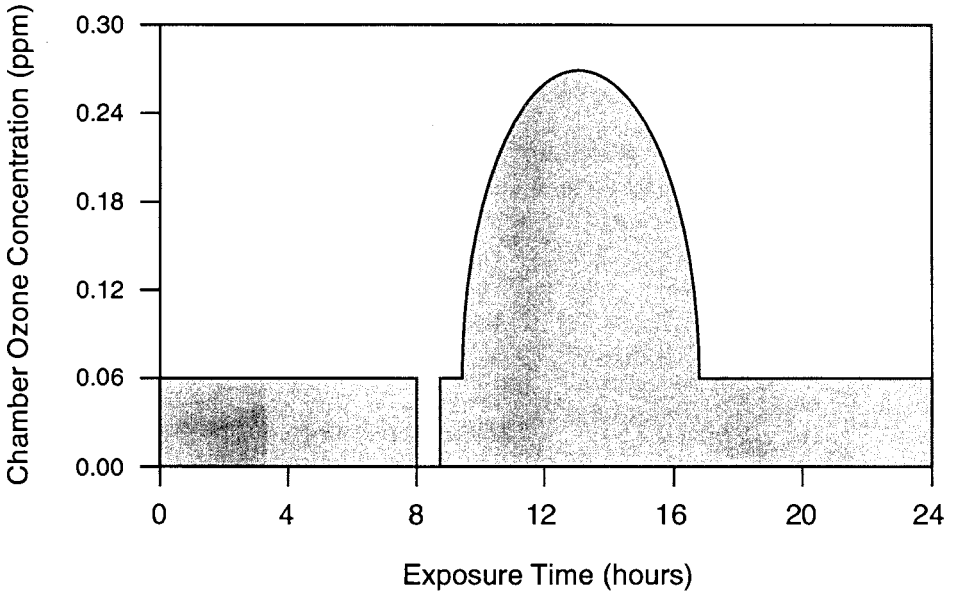

Figure 2. Depiction of a 24-hour O_3 exposure pattern. Mice were exposed to O_3 5 days per week using this pattern of variation in O_3 level. The same mice were exposed continuously to 0.06 ppm O_3 during weekends.

were also recorded. The Dasibi model 1003-AH was calibrated regularly against a standard-source Dasibi model 1008-PC. Standard 8-hour exposures to 0.26 ppm O_3 (peak of exposure pattern) were done Monday through Friday between 0830 and 1630; mice were exposed to 0.06 ppm O_3 for the remaining 16 hours of each exposure day (Figure 2). On weekends, animals were exposed continuously to 0.06 ppm O_3. Chambers were cleaned and food and water replaced on a daily basis; this work took about 30 minutes to complete.

TISSUE PREPARATION AND HISTOLOGIC ANALYSES

Lungs

The lungs were excised and subsequently fixed by perfusion through the trachea with Carnoy's fixative at a constant pressure of 25 cm H_2O. After 2 hours of perfusion and fixation, the lungs were immersed in a large volume of the same fixative. The right caudal lobes were then removed and cut sagitally into 3 pieces. Tissues were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin.

Nasal Cavities

The head of each mouse was removed from the body, depelted, and fixed in Carnoy's fixative as described for the lungs. After fixation, the heads were decalcified in 13% formic acid for 2 days. A tissue block 2 μm to 3 μm thick was cut from the anterior nasal cavity of each mouse by making 2 transverse cuts perpendicular to the hard palate, with each placed at a specific gross anatomic location previously described for rats (Young 1981). The first cut was immediately posterior to the upper incisor teeth, and the second cut was at the incisor papillae of the nasal palate. The reader is referred to either Hotchkiss and associates (1989) or Young (1981) for an illustration of the sampling sites. Tissue blocks were embedded in paraffin. Sections (5 μm) from the anterior surface of the nasal tissue block for each mouse were cut and stained with hematoxylin and eosin.

Serial sections from both lung and nasal tissues were immunohistochemically stained for BrdU as described by Johnson and coworkers (1990) and then counterstained with hematoxylin and lithium carbonate. All slides were coded and counted without knowledge of the treatment group or O_3 exposure. Sections were imaged on a digitizing tablet by camera lucida. Using a microscope at 400× magnification, all BrdU-labeled and nonlabeled epithelial cells were counted throughout each section. The linear length of the basal lamina was calculated from the contour tracing with use of a Sigma Scan. The data for each group were expressed as the mean number of BrdU-labeled nuclei divided by total cells ± SEM. BrdU labeling, when expressed as the number of BrdU-labeled nuclei per millimeter of basal lamina (data not shown) was also measured and led to similar conclusions.

In the nasal cavity, the transitional epithelium lining the medial and lateral surfaces of the maxilloturbinates and the lateral wall was the focus of study because it has previously been shown to have the most significant epithelial damage in the upper airways after O_3 exposure (Hotchkiss et al.

1989). Likewise, terminal bronchioles were the focus of study in the lungs because O_3 exposure causes histologically evident epithelial lesions in these areas (Hotchkiss et al. 1989). A terminal bronchiole was defined as the first branch of a conducting airway proximal to the alveolar ducts. The airway diameters of the terminal bronchioles averaged approximately 100 μm in size. The bronchiolar airway is bounded by a continuous wall that is lined by a cuboidal epithelium. Only terminal bronchioles that were continuous with the proximal alveolar ducts in the section of tissue were used to determine BrdU labeling. The epithelium lining the luminal surface of the bronchioles was identified based on the presence of a basal lamina supporting the cuboidal cells. No distinction was made between nonciliated and type 2 pneumocytes, and luminal macrophages and interstitial fibroblasts were not included in the counts of BrdU-labeled cells. At least 10 terminal bronchioles/lung section and at least 3 sections/mouse were counted. The sample size for these studies was 5 mice from each genotypic group for each exposure on each sample date.

BRONCHOALVEOLAR LAVAGE, TOTAL PROTEIN CONCENTRATION, AND CELL PREPARATION

Mice were killed by cervical dislocation, and the lungs were lavaged in situ 4 times with Hanks' balanced salt solution (HBSS) (35 mL/kg; pH 7.2 to 7.4). The HBSS contains the following (as g/L): NaCl, 8.0; KCl, 0.4; KH_2PO_4, 0.06; $NaHPO_4$, 0.05; $NaHCO_3$, 0.35; dextrose, 1.0. Recovered BAL fluid was immediately cooled at 4°C. For each mouse, the 4 BAL return samples were centrifuged (500 × *g* at 4°C). In addition, the supernatant from the first lavage return was decanted. The total protein concentration in the supernatant was measured and used as an indicator of lung permeability. Numerous indicators of altered air space–blood barrier function have been utilized in oxidant toxicity studies, and each has its limitations. Use of total BAL protein is limited by the uncertain origin of the various proteins (including albumin, immunoglobulins, and enzymes) that are found in BAL return fluid. It is likely, however, that most of the added protein that accumulates after oxidant challenge originates from the serum (Hu et al. 1982), and measurement of total BAL protein is convenient, rapid, and reproducible. A bovine serum albumin protein assay kit (Pierce, Rockford, IL) was used to measure BAL protein. The assay follows the method of Bradford (1976) and is accurate from 10 to 2,000 μg/mL.

The cell pellets from all 4 lavages were combined and resuspended in 1 mL HBSS. Cells were counted with a hemocytometer, and results were expressed as the number of cells recovered in the total BAL return volume. Aliquots (10 mL) were cytocentrifuged (Shandon Southern Instruments, Pittsburgh, PA), and the cells were stained with Diff-Quik (AHS del Caribe, PR) for differential cell analysis. Differential cell counts were done by identifying 300 cells according to standard cytologic techniques. Epithelial cells were identified by the presence of cilia. Because nonciliated cells were not counted, it is therefore likely that epithelial cell loss after exposure is underestimated. Sample sizes for these studies ranged from 4 to 6 mice from each genotypic group for each exposure on each sample date.

VENTILATORY FUNCTION BY WHOLE BODY PLETHYSMOGRAPHY

General Method

Whole body plethysmography as described by Drorbaugh and Fenn (1955) has been used extensively to measure breathing frequency (*f*) and tidal volume (V_T) in small laboratory animals. Its theoretical basis relies on the physical principle that, within a closed chamber, the volume of air inspired will expand as the air is warmed and wetted and will contract upon expiration as the air is cooled and dried. This change in air volume can be measured as a change in pressure. This physical principle has been verified by both theoretical (Epstein and Epstein 1978) and empirical analyses (Chapin 1954). Although the accuracy of the absolute V_T measurement may be questioned, our interests were related primarily to relative changes in V_T, and this method is well suited for these purposes. During the measurement of *f* and V_T, the animal was contained inside a 600 mL plastic tube (length 19.5 cm; inner diameter 6.3 cm) on a platform set above wetted gauze. The tube was enclosed by two rubber stoppers, each with openings to allow air to pass through the barometric chamber. At the top of the chamber, three 0.4 cm ports enabled measurement of pressure, temperature, and gas composition inside the chamber. Changes in air volume were measured with use of a calibrating syringe. Changes in pressure were measured with use of differential pressure transducers (Model 8510B-2, Endevco Co., San Juan Capistrano, CA) and were recorded on a Grass polygraph. Temperature was measured with a type-T thermocouple; chamber air was analyzed for percentage of O_2 and CO_2 prior to and following each measurement of *f* and V_T with use of a mass spectrometer (Model 1100, Perkin-Elmer, Norwalk, CT). Because each measurement of *f* and V_T required the animal to be completely quiescent, a 30- to 60-minute acclimation period preceded the initiation of any testing.

Compressed air (90% relative humidity) was passed through the barometric chamber during the acclimation period at a flow rate of approximately 300 mL/minute. We measured the relative humidity using a wet bulb and dry bulb temperature determination, during which the baro-

metric chamber was open with an air flow of approximately 300 mL/min passing through the chamber. Humidification was performed at the same temperature as the animal chamber during the determinations of relative humidity and during measurements of V_T. After the animal was quiescent, the chamber was sealed by clamping the tubing that extended from the rubber stoppers at both ends. Changes in air pressure were measured for at least 6 seconds, during which the air was analyzed for O_2 and CO_2 and temperature was recorded (± 0.2°C) on the polygraph. Conditions within the chamber during the determination of f and V_T were considered isothermal as evident by a relatively constant baseline tracing. Calculation of V_T was made with the assumption of 90% relative humidity. Each animal was weighed following each test, and calibration of the pressure transducers was performed with a 50 mL gastight syringe at the temperature at which the ventilatory measurements had been made. These protocols have been utilized successfully in previous studies (Tankersley et al. 1993; Paquette et al. 1994).

Data Acquisition and Analysis

The analog signal generated from the pressure transducer was also recorded in digital form with use of a data-acquisition system (Keithley Instruments, Taunton, MA) and a dedicated computer. Data were acquired at an input frequency of 100 Hertz (Hz), and peak inspiration and expiration were determined from approximately 15 consecutive tidal breaths. On the rare occasion during which data were not secured by computer, f and V_T were estimated from 4 tidal breaths recorded on a 6-second strip-chart recording, as described previously (Tankersley et al. 1993). Least squares regression analysis was used to compare the two methods of acquiring ventilatory data, and suitable reproducibility (r^2 = 0.99) was established for each ventilatory measurement. In computation of V_T, the amplitude of the inspiratory and expiratory limbs of each tidal breath was averaged, with the assumption that the body temperature of each animal was constant at 37°C. Minute ventilation ($\dot{V}_E$) was calculated as the product of f and V_T. Ventilatory responses to hypercapnic hypoxia were calculated after a 3- to 5-minute exposure to 8% CO_2 and 10% O_2 balanced with N_2 gas. Each challenge of hypercapnic hypoxia was preceded by and followed by a normal air exposure. The ventilatory measurements in the three groups of mice (+/+, Kit^W/Kit^{W-v}, and Kit^W/Kit^{W-v}-BMT) were highly reproducible, as we have found in other studies with inbred and hybrid mice (Tankersley et al. 1994, 1997). Sample sizes for these studies ranged from 4 to 6 mice from each group for each exposure on each sample date.

EXPERIMENTAL PROTOCOL

The hypothesis that mast cells contribute to the inflammation, epithelial injury and epithelial cell proliferation, and altered ventilatory function induced by subchronic and chronic O_3 exposure was tested as follows. Bone marrow cells were transplanted from mast cell–sufficient (+/+) donor mice to mast cell–deficient (Kit^W/Kit^{W-v}) recipients as described in Bone Marrow Transplantation. Sham transplantation was done in age- and gender-matched +/+ and Kit^W/Kit^{W-v} mice. When peripheral tissues of Kit^W/Kit^{W-v}-BMT mice were repleted with mast cells 16 weeks after transplantation, mice from all three experimental groups were exposed intermittently to O_3 or to filtered air for 1, 3, 14, 30, or 90 days (Figure 3). On the basis of age at time of BMT, mice entered the exposure chamber at the age of 22 to 24 weeks. Immediately following O_3 or air exposure, ventilatory function was assessed as described in Data Acquisition and Analysis. Following

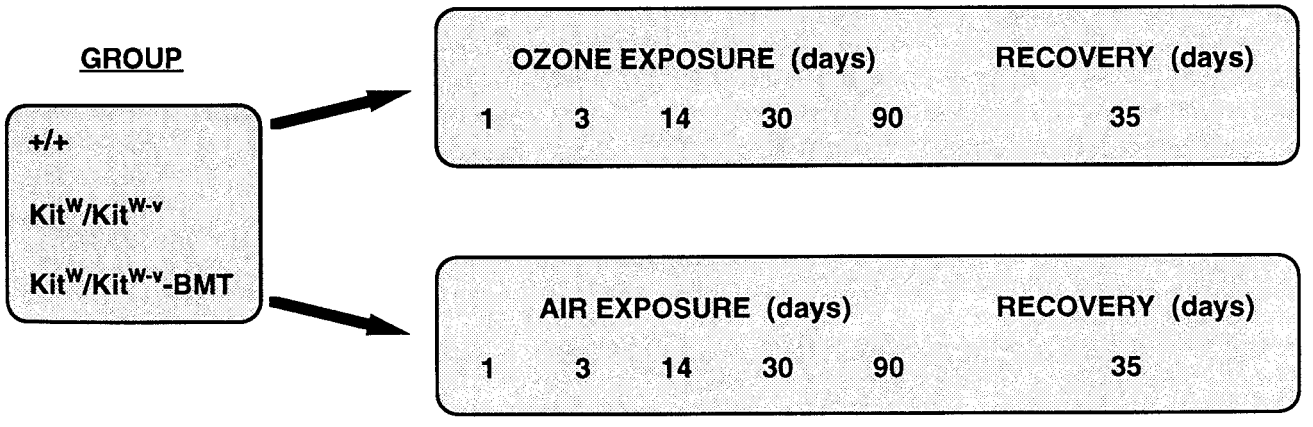

Figure 3. Experimental protocol for O_3 or air exposure. Mice from each experimental group were exposed to O_3 (see Figure 2) or filtered air (0.0 ppm O_3) for up to 90 days. Additional groups of mice from each group were removed from the exposure chambers and allowed to recover for 35 days.

assessment of ventilation, the lungs were evaluated for inflammatory cell infiltration, epithelial sloughing, and hyperpermeability. Epithelial proliferation in the nose and in the centriacinar region of the lungs was also quantitated. To determine whether the effects of subchronic and chronic O_3 exposure were reversible, sets of mice were exposed for 90 days with assessment of nasal and lung injury and ventilatory function conducted after a 35-day recovery period. The durations of exposure and recovery were chosen based on previous studies of subchronic and chronic O_3-exposure effects in rats (Chang et al. 1992) in order to facilitate interstudy and interspecies comparisons. The high frequency of sampling following shorter O_3 exposures reflected the expectation that peak inflammatory response would occur over the first three days of exposure. Ozone- and air-exposure effects on the nose and lungs were compared over time among the three experimental groups of mice. We hypothesized that mast cell–deficient mice would develop significantly less O_3-induced inflammation, tissue injury, and ventilatory dysfunction than would mice with a normal complement of mast cells. The hypothesis was tested by comparing O_3-induced effects in mast cell–deficient mice repleted with mast cells (Kit^W/Kit^{W-v}-BMT) with effects in mast cell–sufficient (+/+) and mast cell–deficient (Kit^W/Kit^{W-v}) mice.

STATISTICAL ANALYSES

Results are expressed as means ± SEM. The effects of exposure (O_3 or air), strain or genotype (+/+, Kit^W/Kit^{W-v}, Kit^W/Kit^{W-v}-BMT), and time duration (1, 3, 14, 30, or 90 days of exposure and 90-day exposure with 35-day recovery period) on dependent variables were assessed by 3-factor analysis of variance (ANOVA). All ANOVA were done with use of the SuperANOVA software package (Abacus Concepts, Inc., Berkeley, CA). The 3 independent variables were exposure, duration, and genotypic group. The dependent variables for analysis were total protein concentration and cells (macrophages, lymphocytes, PMNs, epithelial cells), BrdU uptake (nasal and lung), and ventilatory parameters (f, V_T, $\dot{V}_E$). Sample sizes (repeated measures) were generally 4 to 6 mice per experimental group. Results of these analyses are presented in the appendix. The effects of BMT on mast cell densities in peripheral tissues and on blood parameters were assessed by comparing transplant recipients with age-matched controls by 1-factor ANOVA. Data sets were tested for homoscedasticity as required for parametric analyses, and data that did not meet this requirement (that is, were heteroscedastic) were transformed (natural logarithm [ln] or arsine). Fisher's protected least significant difference (LSD) analyses were used for comparisons of means. Statistical significance was accepted at $p < 0.05$.

RESULTS

In the experiments reported in this section, the O_3 concentration in the exposure chambers did not vary (+/–) from the target concentration by more than 10% during the peak and background exposures, and the Standard Deviation values were less than 2% of the means. As measured at entry and exit from the exposure chambers, there were no differences in body weights among the three genotypic groups. There were also no deaths during the O_3 or air exposures. After mice were removed from the exposure chambers, they were assessed for ventilatory responses to room air and to the challenge of hypercapnic hypoxia. Immediately following these procedures, mice were killed, and one lung from each mouse was lavaged. The opposite lung and the nasal airways were perfused and fixed as described in Methods. The first part of this section describes the effect of subchronic and chronic O_3 exposure on cells recovered by lavage and on airway permeability in +/+, Kit^W/Kit^{W-v}, and Kit^W/Kit^{W-v}-BMT mice. The second part of this section describes O_3- and air-exposure effects on epithelial proliferation in the nasal and centriacinar lung regions in the same animals. The third part compares the effects of subchronic or chronic O_3 exposure on the ventilatory responses in the same three groups of animals. Finally, the fourth part describes blood parameters and mast cell densities in unexposed mice from each genotypic group.

BAL CELL RESPONSES TO FILTERED AIR AND OZONE IN +/+, KIT^W/KIT^{W-v}, AND KIT^W/KIT^{W-v}-BMT MICE

Macrophages

For illustration purposes and because there was no statistically significant effect of duration of air exposure, air controls for each genotypic group were pooled; the means ± SEM are shown in Figure 1 (ANOVA conducted without pooling). There was a statistically significant effect of O_3 exposure on the mean numbers of macrophages recovered by BAL (ANOVA, $p < 0.05$; Appendix Table A.1). The mean number of macrophages recovered from O_3-exposed mice was greater than those recovered from air-exposed mice (Fisher's protected LSD, $p < 0.05$). Furthermore, the mean numbers of macrophages from +/+ and Kit^W/Kit^{W-v}-BMT mice were not different from each other but were significantly ($p < 0.05$) greater than the mean number from Kit^W/Kit^{W-v} mice (Figure 4). There was also a statistically significant effect of time on the macrophage response (Appendix Table A.1),

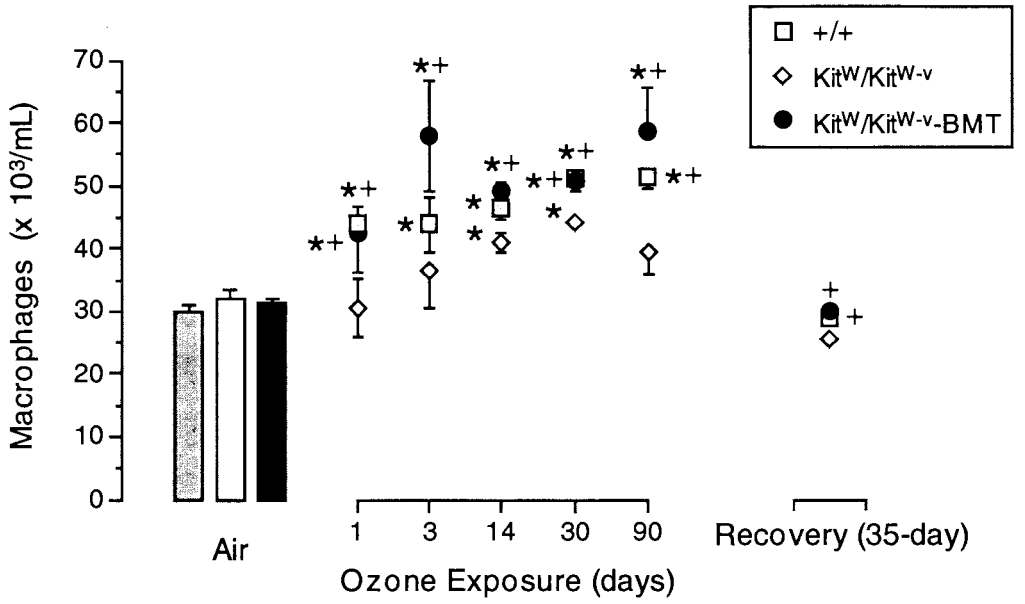

Figure 4. Mean numbers of macrophages recovered by BAL from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT mice after exposure to filtered air or O_3 for up to 90 days and after 35-day recovery. Means ± SEM are presented. Means from air-exposed animals (controls) were not significantly different from each other at any time point and were thus pooled. The sample sizes were 4 to 6 mice per experimental group. Statistical comparison of air vs. O_3: *, $p < 0.05$; comparison of +/+ or Kit^{W}/Kit^{W-v}-BMT with Kit^{W}/Kit^{W-v}: +, $p < 0.05$; comparison of Kit^{W}/Kit^{W-v}-BMT with +/+: #, $p < 0.05$.

as there were increases (Fisher's protected LSD, $p < 0.05$) in the numbers of recovered macrophages up to 14 days of exposure (Figure 4). Macrophages remained elevated in +/+ and Kit^{W}/Kit^{W-v}-BMT mice over the number in air controls throughout the 90-day exposure. However, in mast cell–deficient Kit^{W}/Kit^{W-v} mice, mean macrophage numbers increased relative to those in air controls only after 14- and 30-day exposures to O_3. After a 90-day exposure, macrophage numbers were not statistically different from those in air controls (Figure 4). After a 35-day recovery period from O_3 and air exposure, the mean numbers of macrophages in all three groups of mice returned toward the numbers in air-exposure controls; however, there was a slightly but significantly greater number of macrophages in +/+ and Kit^{W}/Kit^{W-v}-BMT mice compared with Kit^{W}/Kit^{W-v} mice (Figure 4). There was also statistically significant interaction between genotype and exposure, as well as duration and exposure (Appendix Table A.1), which reflects genotype-specific and duration-related changes in macrophage numbers in O_3-exposed animals, with no changes in the respective air-exposed controls.

Lymphocytes

There was a statistically significant effect of O_3 exposure on the mean number of lymphocytes recovered by BAL (ANOVA, $p < 0.05$; Appendix Table A.2). Relative to air controls, the mean numbers of lymphocytes in all three experimental groups of mice were significantly (Fisher's protected LSD, $p < 0.05$) increased after 14 days of O_3 exposure (Figure 5). The numbers of lymphocytes recovered by BAL in +/+ and Kit^{W}/Kit^{W-v} mice returned to respective air-control levels after 30 days of exposure and did not increase again throughout the exposure and recovery periods. The number of lymphocytes recovered from Kit^{W}/Kit^{W-v}-BMT mice was significantly elevated after 30 days of O_3 exposure and returned to baseline thereafter (Figure 5). There was also a significant genotype or "effect of transplantation" (Appendix Table A.2) on the number of BAL-recovered lymphocytes. There were significantly greater numbers of lymphocytes recovered from Kit^{W}/Kit^{W-v}-BMT mice compared with the numbers from +/+ mice ($p < 0.05$), and this difference approached statistical significance when compared with numbers from Kit^{W}/Kit^{W-v} mice ($p = 0.057$). There were no statistically significant differences between the mean numbers of lymphocytes recovered from +/+ and Kit^{W}/Kit^{W-v} mice.

Polymorphonuclear Leukocytes

The numbers of BAL-recovered PMNs were transformed to logarithmic form (ln) to correct for the heterogeneity (heteroscedasticity) of the group variances. There were significant exposure and genotype effects on the mean number of BAL-recovered PMNs in the three experimental groups of mice (ANOVA, $p < 0.05$; Appendix Table A.3). Relative to air exposure, O_3 caused significant infiltration

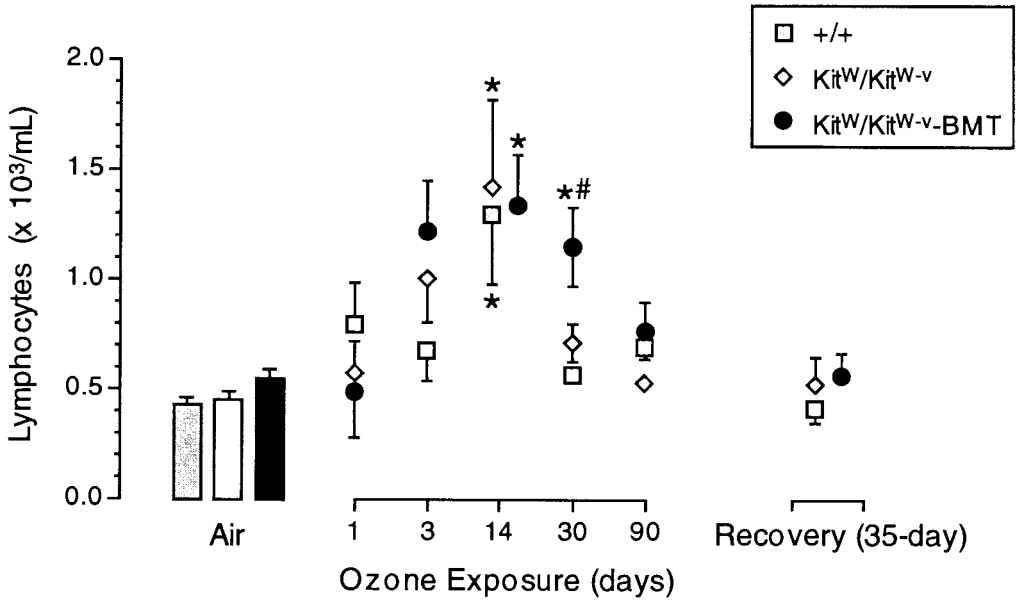

Figure 5. The mean number of lymphocytes recovered by BAL from +/+, Kit^{W}/Kit $^{W-v}$, and KitW/Kit $^{W-v}$-BMT mice after exposure to filtered air or O_3 for up to 90 days and after 35-day recovery. The sample sizes were 4 to 6 mice per experimental group. Symbols are the same as those presented in Figure 4.

of PMNs into the airways of +/+ and Kit^{W}/Kit^{W-v}-BMT mice after exposures of 3, 14, and 30 days (Figure 6). The peak of the response in both groups occurred after 3 days of O_3. There was no statistically significant effect of O_3 exposure on PMN infiltration in Kit^{W}/Kit^{W-v} mice (Figure 6). After 90 days of O_3 and a 35-day recovery period, mean numbers of PMNs in all three groups of mice were not different from their respective air controls (Figure 6).

The mean numbers of PMNs recovered from +/+ and Kit^{W}/Kit^{W-v}-BMT mice were not significantly different from each other, and both were statistically significantly greater than the mean number of PMNs from Kit^{W}/Kit^{W-v} mice (Fisher's protected LSD, $p < 0.05$). There was statistically significant interaction of exposure with duration (Appendix Table A.3), which reflects the changes in PMNs over time in O_3-exposed groups without the corresponding

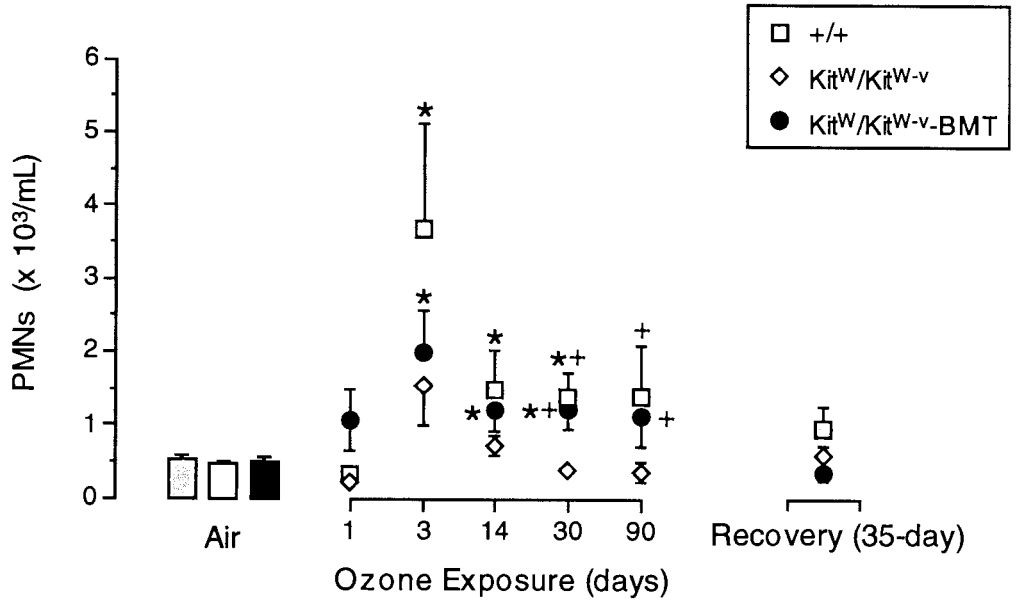

Figure 6. Mean numbers of PMNs recovered by BAL from +/+, Kit^{W}/Kit $^{W-v}$, and Kit^{W}/Kit^{W-v}-BMT mice after exposure to filtered air or O_3 for up to 90 days and after 35-day recovery. The sample sizes were 4 to 6 mice per experimental group. Symbols are the same as those presented in Figure 4.

changes occurring in the air-exposed mice. A significant interaction of exposure and genotype effects (Appendix Table A.3) reflects the lack of O_3-exposure effect in one group (Kit^W/Kit^{W-v}) whereas there were significant O_3-exposure effects in the other groups (+/+, Kit^W/Kit^{W-v}-BMT).

Epithelial Cells

As described for each of the other types of cells recovered by lavage, there were significant genotype, exposure, and duration effects on the mean numbers of epithelial cells recovered by BAL (ANOVA, $p < 0.05$; Appendix Table A.4). Relative to air-exposure, there was a significant effect of O_3 exposure on epithelial cell recovery (Fisher's protected LSD, $p < 0.05$) (Figure 7). The mean numbers of epithelial cells recovered from +/+ and Kit^W/Kit^{W-v}-BMT mice were not significantly different from each other, but both were significantly greater than the number of epithelial cells recovered from Kit^W/Kit^{W-v} mice (Fisher's protected LSD, $p < 0.05$). Within each of the three genotype groups, the greatest number of epithelial cells was recovered after 3 days of O_3 (Figure 7). Numbers declined slightly after 14 days of exposure; however, they remained significantly elevated relative to the numbers from air controls for up to 90 days of O_3 in +/+ and Kit^W/Kit^{W-v}-BMT mice. In Kit^W/Kit^{W-v} mice, epithelial cell numbers returned to air-control levels after 30 days of O_3 and remained at these lower levels throughout the remainder of the O_3 exposure. In all genotype groups, mean epithelial cell numbers were not different from those in the respective air-control mice after a 35-day recovery period (Figure 7). There were also significant interactions among all three independent variables (Appendix Table A.4), which indicated the presence of genotype-specific differences in O_3-exposure effects over time.

Total Protein Concentration

There were statistically significant exposure and duration effects on the total protein concentration in the BAL fluid (ANOVA, $p < 0.05$; Appendix Table A.5). Compared with air exposure, O_3 elicited significant increases in BAL-recovered protein that changed over time (Figure 8). The maximum increases in protein concentration occurred after 3 days of O_3 and returned toward air-control levels thereafter. There were no statistically significant differences between mean total protein concentrations in O_3- and air-exposed animals after 90 days of exposure or after the 35-day recovery period. In contrast to the cellular responses to O_3, there was no statistically significant genotype effect on BAL-recovered protein (Fisher's protected LSD, $p > 0.05$). The absence of differences in protein concentrations between the two genotypic groups—mast cell–sufficient and mast cell–deficient mice—suggests that mast cells do not contribute significantly to the pathogenesis of the lung hyperpermeability induced by O_3 exposure in this study.

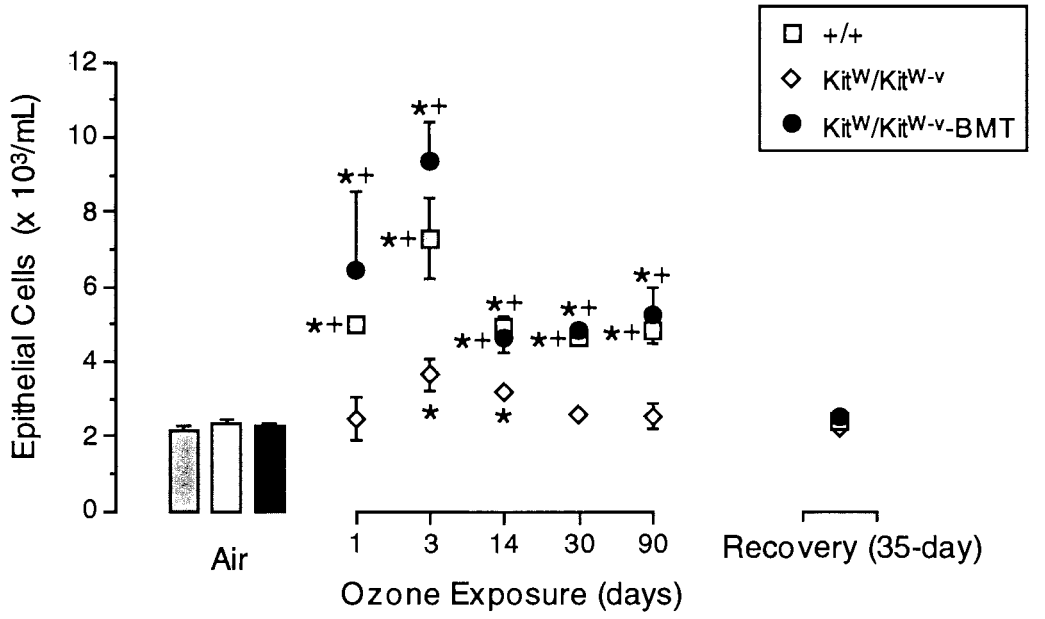

Figure 7. Mean numbers of epithelial cells recovered by BAL from +/+, Kit^W/Kit^{W-v}, and Kit^W/Kit^{W-v}-BMT mice after exposure to filtered air or O_3 for up to 90 days and after 35-day recovery. The sample sizes were 4 to 6 mice per experimental group. Symbols are the same as those presented in Figure 4.

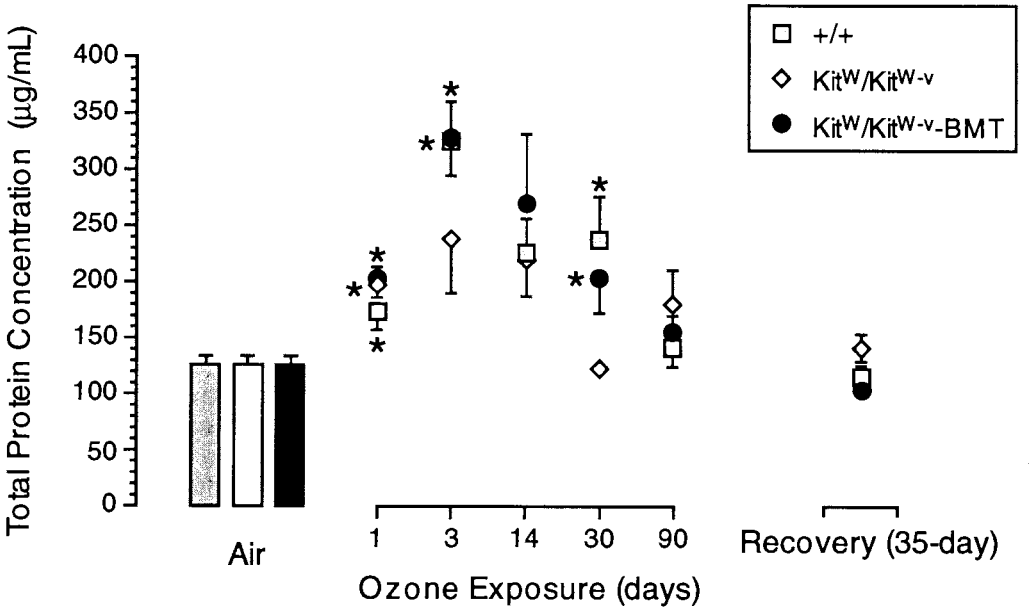

Figure 8. Mean total protein concentration recovered by BAL from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT mice after exposure to filtered air or O_3 for up to 90 days and after 35-day recovery. The sample sizes were 4 to 6 mice per experimental group. Symbols are the same as those presented in Figure 4.

EPITHELIAL CELL PROLIFERATION IN RESPONSE TO FILTERED AIR AND OZONE IN +/+, KIT^{W}/KIT^{W-v}, AND KIT^{W}/KIT^{W-v}-BMT MICE

Lungs

To evaluate the effects of air and O_3 on epithelial cell proliferation in the upper and lower airways (nose and lungs), DNA synthesis was quantitated by counting the number of epithelial cells that incorporated BrdU into nuclei. The numbers of labeled cells were expressed as a function of the length of basal lamina. We focused our study in the centriacinar region of the lung because this anatomical region is particularly susceptible to the oxidant effects of O_3 exposure. All data were transformed into logarithms (ln) for statistical analyses to correct the heterogeneity in variances of the exposure groups. The 3-way ANOVA found statistically significant effects from exposure, genotype, and duration on the mean numbers of BrdU-labeled cells/mm basal lamina (Appendix Table A.6).

In contrast with the findings for cells and protein in the BAL fluid reported above, there was a significant duration effect on the mean number of BrdU-labeled cells in air-exposed mice (Fisher's protected LSD, $p < 0.05$). The peak air effect occurred after 90 days of exposure (Table 1). However, there were no genotype-specific effects of air exposure on BrdU-labeled cells.

Ozone exposure caused significantly greater numbers of BrdU-labeled cells compared with the numbers of labeled cells from mice exposed to filtered air (Fisher's protected LSD, $p < 0.05$). Furthermore, the mean numbers of BrdU-labeled cells were significantly greater in +/+ and Kit^{W}/Kit^{W-v}-BMT mice compared with the numbers in Kit^{W}/Kit^{W-v} mice, and there were no statistically significant differences between labeled cells in +/+ and Kit^{W}/Kit^{W-v}-BMT mice (genotype effect) (Fisher's protected LSD, $p < 0.05$). Significant interaction of genotype effect with the factors of duration and exposure was also found (Appendix Table A.6), indicating that there were genotype-specific effects of exposure over the duration of O_3 exposure.

Relative to air exposure, O_3 caused significantly greater increases in BrdU labeling in +/+ and Kit^{W}/Kit^{W-v}-BMT mice at all time points (Table 1). There was an O_3-exposure effect in Kit^{W}/Kit^{W-v} mice only after 14 days (Table 1). Interestingly, compared with the respective 90-day exposure groups, the mean numbers of BrdU-labeled cells remained significantly elevated (+/+) or increased further (Kit^{W}/Kit^{W-v}-BMT) after the 35-day recovery (Table 1). BrdU labeling in O_3-exposed Kit^{W}/Kit^{W-v} mice was not significantly different from that in air control mice after the 35-day recovery. After recovery, there was occasional mild accumulation of alveolar macrophages in the airway lumens of some centriacinar lung regions of the O_3-exposed +/+ and Kit^{W}/Kit^{W-v}-BMT mice, but no obvious tissue necrosis was detected by light microscopy. No inflammatory cell accumulation or epithelial lesions were evident in tissue distal to the centriacini.

Table 1. Changes (by Duration of Exposure) in Mean BrdU-Labeled Cells[a] in Lung and Nasal Tissues of +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice After Exposure to Air or Ozone

Group[b]	Tissue	Exposure[c]	Duration of Exposure (days) 3	14	30	90	35-Day Recovery
+/+	Lung	Air	1.45 ± 0.25	1.62 ± 0.34	1.08 ± 0.15	1.99 ± 0.58	2.24 ± 0.51
		Ozone	5.42 ± 0.61*	7.85 ± 0.88*	4.93 ± 0.51*	4.27 ± 0.37*	5.20 ± 0.94*
	Nasal	Air	0.14 ± 0.09	0.27 ± 0.20	0.55 ± 0.32	0.35 ± 0.26	0.19 ± 0.14
		Ozone	0.64 ± 0.44*	0.50 ± 0.22	0.26 ± 0.08	2.35 ± 1.06	0.76 ± 0.47*
Kit^{W}/Kit^{W-v}	Lung	Air	1.33 ± 0.26	1.68 ± 0.26	1.13 ± 0.16	3.40 ± 0.81	3.46 ± 1.02
		Ozone	2.28 ± 0.49	3.23 ± 0.41*	1.22 ± 0.16	4.97 ± 1.11	3.98 ± 1.19
	Nasal	Air	0.24 ± 0.11	0.45 ± 0.17	0.49 ± 0.32	2.04 ± 0.75	0.23 ± 0.11
		Ozone	0.40 ± 0.33	1.70 ± 0.50*	0.32 ± 0.21	2.86 ± 0.90	0.38 ± 0.30
Kit^{W}/Kit^{W-v}-BMT	Lung	Air	1.57 ± 0.28	1.44 ± 0.22	1.56 ± 0.22	3.56 ± 0.88	2.36 ± 0.58
		Ozone	3.43 ± 0.34*	3.47 ± 0.52*	3.96 ± 0.29*	9.48 ± 1.70*	11.60 ± 0.86*
	Nasal	Air	0.20 ± 0.08	0.29 ± 0.15	0.27 ± 0.20	0.42 ± 0.33	0.59 ± 0.18
		Ozone	0.60 ± 0.17*	0.52 ± 0.30	0.31 ± 0.31	0.71 ± 0.19	1.28 ± 0.36*

[a] Data are presented as mean ± SEM number of BrdU-labeled cells per mm of basal lamina. Details of the exposure regimen are given in the text. An asterisk (*) indicates a significant ($p < 0.05$) difference between results for air and ozone exposures.

[b] $n = 4$ to 6 mice in each experimental group.

[c] Where indicated, mice were exposed to 0.26 ppm O_3 8 hours/day for the indicated number of days.

Nasal Cavities

The transitional epithelium lining the medial and lateral surfaces of the maxilloturbinates and the lateral wall was the focus of study for air- and O_3-exposure effects. Cell proliferation data were transformed into logarithms (ln) for statistical analyses to correct the heterogeneity in variances of the exposure groups. Compared with the proliferative responses observed in lung tissue, the responses to O_3 exposure in the nose were slight, although statistically significant. Exposure and duration effects on the mean number of BrdU-labeled cells were found in the 3-way ANOVA ($p < 0.05$), but the effect of genotype was not statistically significant (Appendix Table A.7). There were no obvious inflammatory lesions or epithelial damage in the nasal airways in any of the genotypic groups at any time point of duration.

The mean number of labeled epithelial cells in the nasal airways increased significantly in the air-exposed animals of each genotypic group up to 90 days of exposure and then declined after the 35-day recovery period (Fisher's protected LSD, $p < 0.05$; Table 1). There were no statistically significant differences among genotypic groups at any time point after air exposure. Relative to the number of labeled cells in respective air controls, there was a significant increase in the number of labeled cells in +/+ and Kit^{W}/Kit^{W-v}-BMT mice, but not in Kit^{W}/Kit^{W-v} mice, after the 35-day recovery from O_3 (Table 1).

VENTILATORY RESPONSE TO FILTERED AIR AND OZONE IN +/+, KIT^{W}/KIT^{W-v}, AND KIT^{W}/KIT^{W-v}-BMT MICE

Baseline Breathing

Statistically significant effects of genotype and exposure were found on frequency of baseline breathing (that is, breathing of room air) in the three groups of mice (ANOVA, $p < 0.05$; Appendix Table A.8). Following filtered-air exposure, the breathing frequency of Kit^{W}/Kit^{W-v} mice was different from that of +/+ and Kit^{W}/Kit^{W-v}-BMT mice (Fisher's protected LSD, $p < 0.05$) (Figure 9). Following 1 day of O_3, the breathing frequency in Kit^{W}/Kit^{W-v} and Kit^{W}/Kit^{W-v}-BMT mice was significantly more rapid compared with the frequency in +/+ mice, which was slightly reduced compared with the frequency in air controls (Figure 9). These results suggest that the transfer of bone marrow cells to Kit^{W}/Kit^{W-v} mice did not reverse the O_3-exposure effect on breathing frequency (or on minute ventilation, as will be discussed). However, there was no effect of O_3 in any of the three groups compared with their respective air controls after 14 days of exposure, and no further effects of O_3 were detected through the full 90-day exposure period. The significant interaction between exposure and duration (Appendix Table A.8) reflects the duration-specific effects of O_3 and filtered air on breathing frequency (that is, exposure effects seen early in the experiment that diminished over

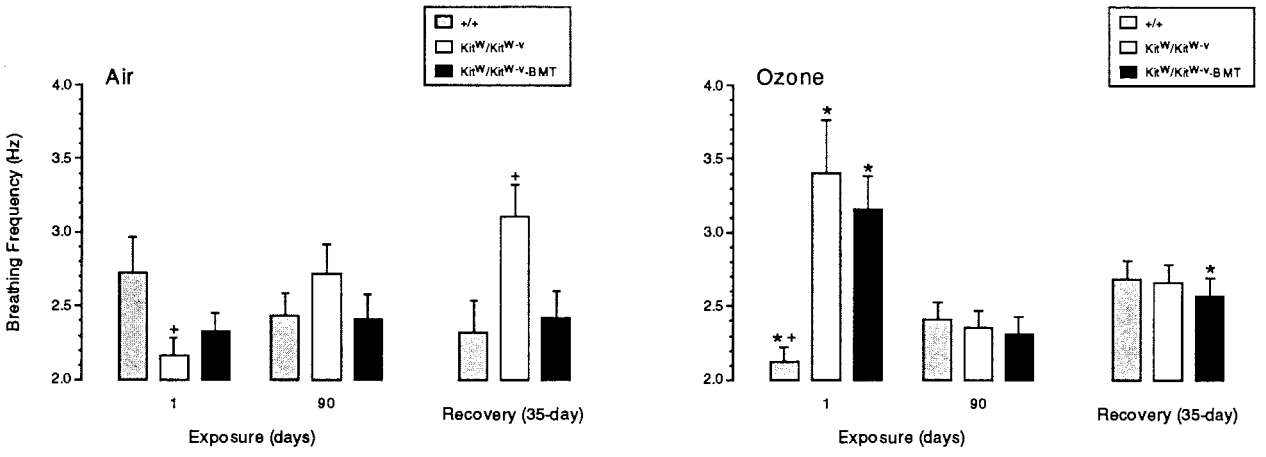

Figure 9. Breathing frequency (f, Hz) of +/+, Kit^W/Kit^{W-v}, and Kit^W/Kit^{W-v}-BMT mice breathing room air after exposure to filtered air (left) or O_3 (right). The sample sizes were 4 to 6 mice per experimental group. Symbols are the same as those presented in Figure 4.

time). Note that because genotype and exposure effects were found only after 1 day and 90 days of exposure and after a 35-day recovery, we have included in Figures 10, 11, and 12 only those data in order to clarify presentation.

There were no statistically significant effects of genotype or exposure on mean tidal volume (V_T), although there was an effect of duration (Figure 10; Appendix Table A.9). The duration effect is likely to be a consequence of body growth during the 4-month exposure period. After 1 day of exposure to filtered air, the tidal volume of +/+ mice was significantly greater than the tidal volumes of both Kit^W/Kit^{W-v}-BMT and Kit^W/Kit^{W-v} mice (Figure 10). There were no differences among the air-exposed groups afterward. Furthermore, there were no differences among the 3 experimental groups after exposure to O_3 or after 35-day recovery (Figure 10).

An estimate of $\dot{V}_E$ in each mouse was calculated as the product of f and V_T. Because there was little difference in V_T among the experimental groups, changes in $\dot{V}_E$ were largely driven by changes in f (as depicted in Figures 9 and 11). There were no detectable effects of genotype, duration, or exposure on $\dot{V}_E$ (Appendix Table A.10), although exposure effect approached statistical significance ($p = 0.066$). Significant interaction among genotype, exposure, and duration indicates that there was a complex interplay of these factors on $\dot{V}_E$ in this study (Appendix Table A.10). The majority of the interaction occurred after 1 day of exposure, when differential effects of O_3 and air exposure were observed with all three experimental groups of mice. At that point, O_3 caused rapid breathing (with increased $\dot{V}_E$) in Kit^W/Kit^{W-v}-BMT and Kit^W/Kit^{W-v} mice and caused decreased breathing frequency (with decreased $\dot{V}_E$) in the +/+

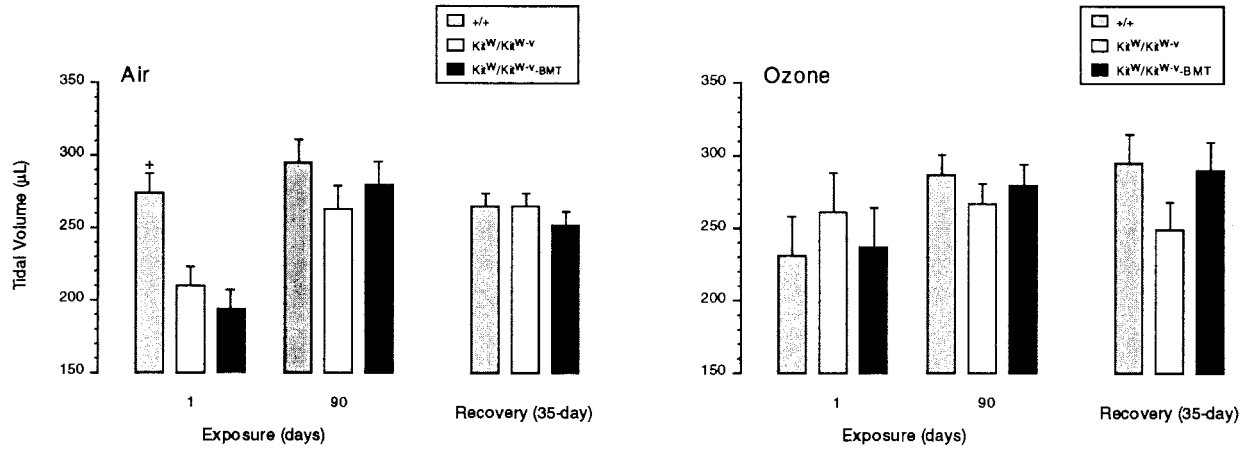

Figure 10. Tidal volume (µL) of +/+, Kit^W/Kit^{W-v}, and Kit^W/Kit^{W-v}-BMT mice breathing room air after exposure to filtered air (left) or O_3 (right). The sample sizes were 4 to 6 mice per experimental group. Symbols are the same as those presented in Figure 4.

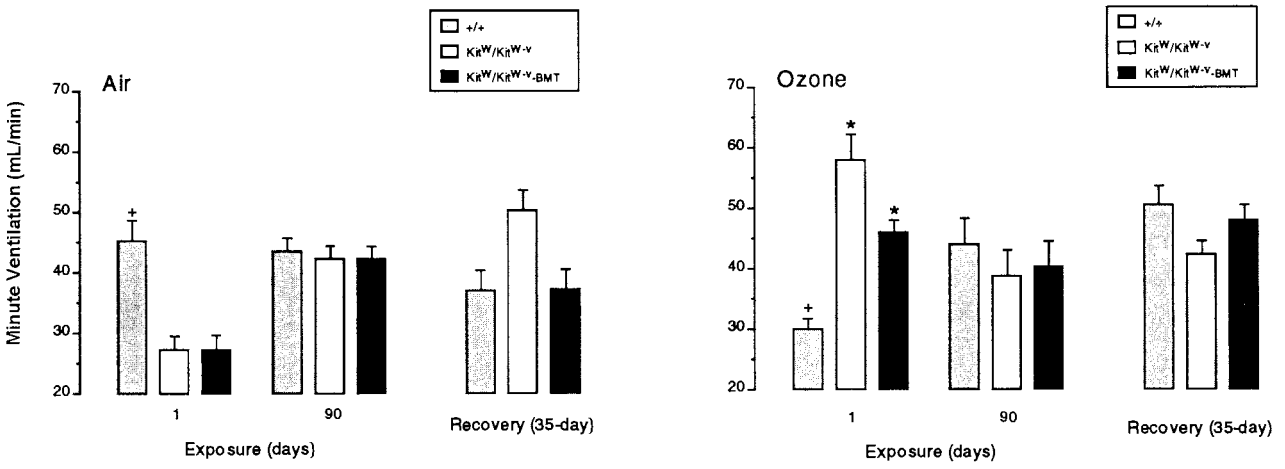

Figure 11. The minute ventilation (mL/min) of +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT mice breathing room air after exposure to filtered air (left) or O_3 (right). The sample sizes were 4 to 6 mice per experimental group. Symbols are the same as those presented in Figure 4.

mice (Figures 9 and 11). Subsequent to 1 day of exposure, there was apparent adaptation of the ventilatory responses ($\dot{V}_E$ and f) to O_3 in all three groups of mice.

Response to Hypercapnic Hypoxia

Each mouse was challenged with hypercapnic hypoxia (8% CO_2, 10% O_2; see Methods), and the change in $\dot{V}_E$ (percent of $\dot{V}_E$ response to room air) was measured to provide a noninvasive evaluation of the effects of exposure and genotype on ventilatory function. There were no statistically significant effects of genotype, exposure, or duration on the mean $\dot{V}_E$ response to hypercapnic hypoxia, although the effect of genotype approached statistical significance ($p = 0.066$) (Appendix Table A.11). There was significant interaction among all 3 factors on $\dot{V}_E$ response to hypercapnic hypoxia (Appendix Table A.11). Interestingly, the response to this challenge was significantly depressed in +/+ mice relative to that in Kit^{W}/Kit^{W-v}-BMT and Kit^{W}/Kit^{W-v} mice after 1 day of filtered-air exposure (Figure 12). Subsequent to this time point, there were no major differences among the 3 groups, although the mean response in Kit^{W}/Kit^{W-v}-BMT mice was significantly greater than that in Kit^{W}/Kit^{W-v} mice after a 35-day recovery period in filtered air (Figure 12). After 1 day of O_3 exposure, the ventilatory response to hypercapnic hypoxia was increased in +/+ mice and decreased in Kit^{W}/Kit^{W-v}-BMT and Kit^{W}/Kit^{W-v} mice. Furthermore, the mean ventilatory response to hypercapnic hypoxia in +/+ mice was significantly different from the mean responses in Kit^{W}/Kit^{W-v}-BMT and Kit^{W}/Kit^{W-v} mice. As observed in the air-exposed animals, the effects of O_3 on ventilatory responses to challenge with hypercapnic hypoxia were largely attenuated over exposure duration, although the mean ventilatory response in Kit^{W}/Kit^{W-v}-BMT ice was significantly elevated relative to +/+ (90 days) and Kit^{W}/Kit^{W-v} (35-day recovery) mice.

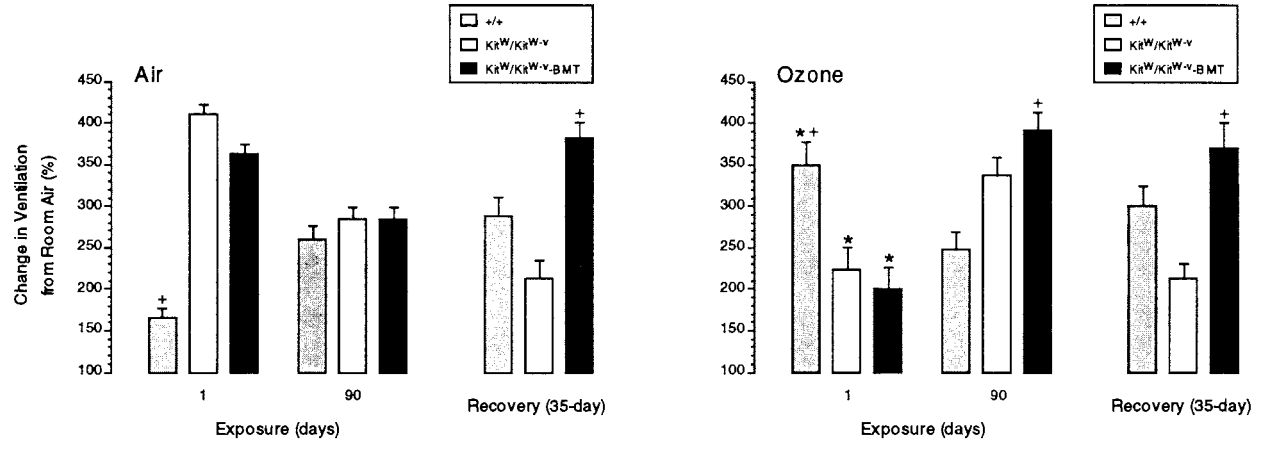

Figure 12. The minute ventilation response (mL/min) of +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT mice to challenge of hypercapnic hypoxia after exposure to filtered air (left) or O_3 (right). Responses are expressed as the percent of $\dot{V}_E$ response to room air. Composition of gases for hypercapnic hypoxia: $FiCO2 = 0.08$ and $FIO_2 = 0.10$. The sample sizes were 4 to 6 mice per experimental group. Symbols are the same as those presented in Figure 4.

Table 2. Blood Characteristics of 25-Week-Old +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice[a]

Group[b]	Red Blood Cells (M/μL)[c]	Hematocrit (%)	Hemoglobin (mEq/L)	White Blood Cells (K/μL)[d]	Platelets (K/μL)[d]
+/+	9.8 ± 0.6	47.8 ± 0.2	14.2 ± 0.2	9.9 ± 1.0	1,497 ± 65
Kit^{W}/Kit^{W-v}	6.9 ± 0.2*	36.9 ± 0.6*	12.0 ± 0.1*	6.6 ± 0.9*	1,282 ± 18*
Kit^{W}/Kit^{W-v}-BMT	10.8 ± 0.4	46.3 ± 0.9	14.1 ± 0.2	9.0 ± 0.1	1,184 ± 63*

[a] Data are presented as mean ± SEM. An asterisk (*) indicates a significant ($p < 0.05$) difference from the +/+ group.

[b] $n = 5$ to 7 mice in each group.

[c] Millions of red blood cells per μL whole blood.

[d] Thousands of white blood cells or platelets per μL whole blood.

BLOOD CHARACTERISTICS AND PERIPHERAL TISSUE MAST CELL DENSITIES IN +/+, KIT^{W}/KIT^{W-v}, AND KIT^{W}/KIT^{W-v}-BMT MICE

There was a statistically significant genotype effect on both mean percent hematocrit and hemoglobin concentration, as well as numbers of red blood cells, white blood cells, and platelets (ANOVA, $p < 0.05$). The means of each of these parameters were significantly greater in +/+ mice than in age-matched Kit^{W}/Kit^{W-v} mice (Fisher's protected LSD, $p < 0.05$) (Table 2). With the exception of platelets, each of these deficits in blood parameters was corrected by BMT from +/+ to Kit^{W}/Kit^{W-v} mice (Table 2).

There was also a statistically significant effect of genotype on the mean number of mast cells in tracheas and mainstem bronchi of the three groups of mice (ANOVA, $p < 0.05$). In both tissues, the density of mast cells was significantly greater in +/+ mice than in age-matched Kit^{W}/Kit^{W-v} mice, in which no mast cells were found (Fisher's protected LSD, $p < 0.05$) (Table 3). Bone marrow transplantation restored mast cell densities in mainstem bronchi, and there were no significant differences between +/+ and Kit^{W}/Kit^{W-v}-BMT mice (Table 3). Mast cell densities were also significantly increased in the tracheas of Kit^{W}/Kit^{W-v}-BMT mice compared with densities in Kit^{W}/Kit^{W-v} animals, although the densities were still less than those in the +/+ group (Table 3).

DISCUSSION

The overall objective of this study was to test the hypothesis that mast cells contribute significantly in the initiation and propagation of pathological airway responses to ozone exposure. To accomplish this objective, we employed the use of a mast cell–deficient mouse model as characterized in the Introduction. In order to simplify discussion of the results of these experiments, we have generated a table (Table 4) that summarizes the major findings on 3 components of the response to ozone: inflammation, epithelial cell proliferation, and ventilatory function.

A pattern of O_3 exposure that had a peak level of 0.26 ppm and a background level of 0.06 ppm O_3 was utilized in this study. The exposure pattern was designed to mimic a pattern of O_3 production in heavily populated urban environments, in which O_3 concentrations gradually increase during morning hours and peak concentrations may exceed 0.26 ppm before decreasing later in the afternoon (Lippmann 1989). It is important to note that the actual delivered dose of O_3 to the lungs of rodents may be considerably less than may be expected by routine calculation (namely, concentration × time) in human subjects because rodents are obligate nose-breathers. Accordingly, their nasal airways are very efficient at removing toxicants from inhaled air. Empirical support for the enhanced scrubbing efficiency of the upper airways of rodents compared with the upper airways in humans comes from Hatch and coworkers (1994). Using isotope of oxygen (^{18}O)-labeled O_3, these investigators found that the amount of O_3 that reaches the bronchiolar-alveolar regions of resting rats exposed to 2.0 ppm O_3 for 2 hours was less than the amount of ^{18}O incorporated into the homologous regions of exercising human subjects exposed to 0.4 ppm O_3 for 2 hours. Therefore, the dose of O_3 delivered to the lungs of mice in our exposure protocol is almost certainly considerably less than would occur in human subjects given similar O_3 exposure.

RESPONSES TO OZONE EXPOSURE IN +/+ MICE

Inflammation

In mast cell–sufficient +/+ mice, the O_3 profile exposure initiated an inflammatory response in the lungs after 1 day of exposure that was characterized by significant infiltration of macrophages into airways, sloughing of epithelial cells, and lung hyperpermeability, with the last defined

Table 3. Mast cells in Tracheal and Mainstem Bronchial Tissues of 25-Week-Old +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice[a]

Group[b]	Tissue Sampled	Mast Cells/mm^2 [c]
+/+	Trachea	79 ± 7
	Mainstem bronchi	68 ± 5
Kit^{W}/Kit^{W-v}	Trachea	0 ± 0*
	Mainstem bronchi	0 ± 0*
Kit^{W}/Kit^{W-v}-BMT	Trachea	52 ± 8*
	Mainstem bronchi	84 ± 9

[a] Data are presented as mean ± SEM. An asterisk (*) indicates a significant ($p < 0.05$) difference from the +/+ group.

[b] $n = 5$ to 7 mice in each group.

[c] Mast cells closely associated with the epithelium (within or in juxtaposition to the basal surface of the epithelium) were counted and expressed as the number of mast cells per mm^2 of epithelium.

Table 4. Summary of Ozone Exposure Effects on Cells in BAL Fluid, Epithelial Cell Proliferation, and Ventilation in +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice[a]

	Group					
Parameter	+/+		Kit^{W}/Kit^{W-v}		Kit W/Kit^{W-v}-BMT	
BAL						
Macrophages	⇑⇑⇑;	no return; recovery (35 days to post)	⇑;	partial return (by 90 days); recovery	⇑⇑⇑;	no return; recovery (35 days post O_3)
Lymphocytes	⇑;	full by 30 days)	⇑;	full return (by 30 days)	⇑;	full return (By 90 days)
PMNs	⇑⇑⇑;	partial (by 14 days); recovery (35 days)	⇔		⇑⇑;	partial return (by 90 days); recovery (35 days post O_3)
Epithelial cells	⇑⇑;	partial return (by 14 days); recovery (35 days post O_3)	⇑;	full return (by 30 days)	⇑⇑⇑;	partial return (by 14 days); recovery (35 days post O_3)
Protein	⇑⇑⇑;	full return (by 90 days)	⇑;	full return (by 3 days)	⇑⇑⇑;	full return; recovery (by 90 days)
BrdU Uptake						
Nasal	⇑;	partial return (by 14 days) no recovery	⇑;	full return (by 30 days)	⇑;	partial return (by 14 days); no recovery
Lungs	⇑⇑⇑;	partial return (by 30 days); no recovery	⇑;	full return (by 30 days)	⇑⇑;	no return; no recovery
Ventilation						
Room air	⇔		⇑;	full return (by 14 days)	⇑;	full return (by 14 days)
Hypercapnic hypoxia	⇑	full return (by 14 days)	⇓;	full return (by 14 days)	⇓;	full return (by 14 days)

[a] Arrows indicate the magnitude and direction of response compared with respective air-exposed control animals. Return indicates the tendency to return to air control levels during exposure. Recovery indicates the response 35 days after O_3 exposure ended compared with respective air-exposed control animals.

by increased total protein concentration in BAL samples. After 3 days of exposure, PMNs began to infiltrate into airways, and macrophage number and total protein concentration continued to increase. By 14 days of exposure, PMNs, epithelial cells, and total protein began to return toward baseline, whereas macrophage numbers remained elevated and the number of lymphocytes increased. After 14 days of exposure, mean numbers of PMNs and epithelial cells had declined to plateau levels. Numbers of lymphocytes and total protein concentrations were not different from the values in air controls after 30 and 14 days, respectively. The kinetics of the inflammatory cell responses to O_3 exposure in this genotype of mouse suggest that, although adaptation to O_3 exposure occurred for some cells, the increase in macrophages and PMNs remained and epithelial sloughing continued. After a 35-day recovery period from O_3 exposure, all inflammatory-response parameters returned to levels that were not different from the same parameters in air controls.

The mechanisms underlying this phenomenon of adaptation are unclear, but a number of possibilities exist. For example, the upper airways may have become more efficient scrubbers of the inhaled O_3. Although we detected no obvious characteristics of O_3-exposed upper airways that would enhance scrubbing (characteristics such as increased mucous production or epithelial surface area), it is possible that the antioxidant capacity of the epithelial cells lining the upper airways may have increased. Acute and subacute exposures of rats to O_3 have caused increased activities of whole-lung antioxidant enzymes such as glucose-6-phosphate dehydrogenase, glutathione reductase, and glutathione peroxidase (Chow 1976; Mustafa and Tierney 1978; Bassett et al. 1988). It is possible that similar enhanced production of these and other antioxidant enzymes may have contributed to the patterns of adaptation observed in the present study.

Epithelial Cell Proliferation

The proliferative response of the centriacinar epithelium to O_3 exposure in +/+ mice paralleled the epithelial sloughing detected by BAL. There were significant increases in DNA synthesis as early as 3 days of O_3 exposure (approximately 275% compared with values in air controls), and this level of proliferation was the same or increased up to 30 days of exposure. After 90 days of exposure, proliferation declined in O_3-exposed mice but was still significantly elevated relative to DNA sythesis in air-control mice. There was also an O_3-exposure effect on nasal epithelium, although the magnitude of this response was considerably less than that observed in the lungs. Interestingly, DNA synthesis remained elevated after the 35-day recovery period in both nasal and lung tissues. In other words, in the absence of O_3 and while inflammatory-response parameters (macrophages, PMNs, total protein) declined, increased epithelial cell proliferation continued compared with proliferation rates in air-exposed controls. These results may imply that the signal(s) for epithelial cell proliferation are distinct from those initiating the infiltration of inflammatory cells, although mast cells probably mediate both events. It is important to note a potential limitation in the study. Although a concerted effort was made to identify only epithelial cells, it is possible that some basal cells were also counted. While we believe it unlikely that cell types other than epithelial cells contributed significantly to the reported cell proliferation rates, it is possible that some cells other than epithelial cells were included.

Ventilatory Function

There were minimal effects of prolonged O_3 exposure on ventilatory function in +/+ mice relative to function in air-exposed controls. There was a tendency toward decreased $\dot{V}_E$ in room air after 1 day of exposure to O_3 (due largely to decreased f), but this trend was reversed at all timepoints thereafter. These results agree with those of Tepper and coworkers (1991), who also found slightly decreased $\dot{V}_E$ in rats exposed to O_3 (peak level 0.24 ppm) for up to 78 weeks, but their changes were not statistically significant compared with values in air-exposed controls. However, we found that O_3 caused an unexpected significant increase in the ventilatory response to the challenge of hypercapnic hypoxia in +/+ mice after 1 day of O_3 exposure. In previous studies, we had found that O_3 exposure decreased responses to hypercapnic hypoxia in O_3-susceptible C57BL/6J mice (Tankersley et al. 1993). The addition of hypoxia to the formulation of the challenge gas may have contributed to the differences between the two studies. We demonstrated previously that the combination of hypoxia with hypercapnic challenge in inbred mice elicits greater ventilatory responses than does hypercapnia alone (Tankersley et al. 1994). Tepper and coworkers (1991) also found that chronic O_3 exposure of rats caused decreased responsiveness to hypercapnic challenge. The diminished hypercapnic responses were interpreted as an effect on ventilatory control via alterations in pulmonary chemoreflex and C-fiber stimulation caused either directly by O_3 or indirectly via inflammatory processes. The mechanisms through which O_3 exposure may enhance responsivity to hypercapnic hypoxia are not obvious. In any case, the effect in +/+ mice was attenuated with increased exposure duration, and there were no O_3-exposure effects after 1 day of exposure.

It is interesting to note there was no apparent relation between O_3-exposure effect on functional lung characteristics (that is, ventilation pattern) despite significant inflammatory changes and epithelial injury and proliferation that were occurring over the same period. The dissociation of O_3-induced inflammatory effects and pulmonary-function changes in human subjects has been described previously. Although there are obvious differences in the O_3-exposure regimen and measurement of pulmonary function in the mouse studies compared with human studies, there are some interesting parallels. Basha and coworkers (1994) found significant increases in PMNs and inflammatory mediators in BAL fluid from subjects with asthma after acute exposure to 0.20 ppm O_3, whereas McBride and coworkers (1994) found increases in PMNs and epithelial cells in nasal lavage returns from subjects with asthma exposed to 0.24 ppm O_3. There were no detected changes in pulmonary function in either human study group. Similarly, Balmes and coworkers (1996) and Schelegle and coworkers (1991) found no statistically significant correlation between O_3-induced inflammatory parameters and changes in pulmonary function in healthy normal human subjects. One of the important implications of these studies is that the traditional indicators of O_3-exposure effects in the lungs (that is, spirometric indicators of altered lung function) may not reflect inflammation in the small airways and epithelial cell injury. (However, see Weinmann and coworkers [1995] for discussion of volume-adjusted forced expiratory flow in 0.25–0.75 second [FEF_{25-75}] as a marker of dysfunction in small airways.) The undetected inflammation may lead to chronic airway injury, increasing phenotypic changes or proliferation in epithelial cells. The undetected inflammation may also enhance susceptibility to subsequent challenges.

ROLE OF MAST CELLS IN MEDIATING AIRWAY RESPONSES TO OZONE EXPOSURE

Inflammation

To evaluate the role of mast cells in mediating these responses to O_3 exposure, we compared the inflammation, cell proliferation, and ventilation results in mast cell–deficient Kit^W/Kit^{W-v} mice with those in age-matched, mast cell–sufficient +/+ animals. The procedures of BMT effectively reversed the anemia and peripheral-tissue mast-cell deficit in Kit^W/Kit^{W-v} mice as had been demonstrated previously by our laboratory and by others (Kleeberger et al. 1993b). Whereas O_3 exposure caused an increase in the mean number of BAL-recovered macrophages (at 14 and 30 days of exposure) and epithelial cells (at 3 to 30 days of exposure), but not of PMNs, in Kit^W/Kit^{W-v} mice, the magnitudes of response were significantly less than those of congenic +/+ mice. Furthermore, there was an apparent adaptation in the Kit^W/Kit^{W-v} mice after 90 days of exposure that was not observed in the +/+ mice. These results suggest that mast cells contribute significantly to the pathogenesis of cellular responses to O_3 exposure in this model, but not to the change in lung permeability. To further test this hypothesis, we determined whether the inflammatory responses to O_3 in Kit^W/Kit^{W-v} mice could be reversed with repletion of peripheral-tissue mast cells. There were significant O_3-exposure effects on each of the cell types, and total protein concentration in Kit^W/Kit^{W-v}-BMT mice relative to the parameters in age-matched, air-exposed control animals, and the kinetics of these responses were similar to those in +/+ mice. The magnitudes of response in the Kit^W/Kit^{W-v}-BMT mice closely resembled the responses in +/+ mice; there were no statistically significant differences in BAL-recovered cells and protein in the two groups of mice after O_3 exposure. However, the magnitude of change in numbers of macrophages, PMNs, and epithelial cells was significantly greater in Kit^W/Kit^{W-v}-BMT mice than in Kit^W/Kit^{W-v} animals. There were no differences in O_3-induced lung hyperpermeability between the same two groups. These results provide further support for the hypothesis that mast cells mediate the O_3-induced infiltration of macrophages and PMNs in the lungs, as well as epithelial-cell sloughing. The effect of O_3 on lung permeability appears to be regulated independently of mast cell activity.

These observations are largely consistent with our previous studies, which demonstrated a role for mast cells in lung cell responses to short-term (that is, 3-hour) exposure to 2.0 ppm O_3 (Kleeberger et al. 1993b; Longphre et al. 1996a). In this acute O_3-exposure model, we found that the short-term reversible inflammation, epithelial cell loss, and hyperpermeability induced by such high concentrations of O_3 were significantly less in magnitude in the lungs and nasal airways of mast cell–deficient mice compared with the magnitude in mast cell–sufficient mice. The current study has demonstrated that, in addition to its role in modulating O_3-induced acute inflammation, the mast cell is also important in propagating and maintaining the inflammatory cell infiltration and epithelial loss induced by chronic O_3 exposure. The lack of a detectable effect of mast cells on the permeability response to chronic O_3 exposure may be a consequence of the nature of the two exposure protocols. Other investigators have speculated that acute exposure to high O_3 concentrations may lead to degranulation and secretion by mast cells, whereas chronic exposure to lower O_3 concentrations may lead to selective stimulation of mast cells. That is, mast cells may be induced to secrete qualitatively and quantitatively different profiles by different exposures.

Compared with findings from acute and subacute O_3-exposure models, the magnitude of inflammation and epithelial cell loss induced by the chronic exposure in this model was somewhat smaller. The numbers of PMNs recovered 6 hours after a 3-hour exposure to 2.0 ppm O_3 were $11.6 \pm 1.7 \times 10^3$/mL in +/+ mice and $3.0 \pm 1.2 \times 10^3$/mL in Kit^W/Kit^{W-v} mice (Kleeberger et al. 1993b), which were approximately 3.1- and 2.0-fold greater, respectively, than the maximum numbers of PMNs recovered in the present study. In a subacute O_3-exposure study (Kleeberger et al. 1993a), the numbers of PMNs and epithelial cells recovered from C57BL/6J mice (that have O_3 susceptibility similar to that in $WBB6F_1$-+/+ mice) after 48 to 72 hours of continuous exposure to 0.3 ppm O_3 were 2.7- and 1.5-fold greater than those recovered in the present study. The disparity in magnitudes of inflammation and epithelial cell loss between the models may be due to a number of factors, including the obvious differences in exposure level. Another important factor to consider is the age of the mice. Because 16 weeks were required to restore mast cells to the peripheral tissues of Kit^W/Kit^{W-v}-BMT mice, the mice were approximately 24 weeks of age before O_3 exposures were initiated. In a previous study with this mouse model, we demonstrated that the inflammatory responses induced in 6- to 8-week-old +/+ and Kit^W/Kit^{W-v} mice were significantly greater than those found in 22- to 24-week-old mice. Interestingly, the numbers of PMNs recovered by BAL after acute O_3 exposure of 22- to 24-week-old animals were similar to those in the present study. The importance of age as a susceptibility factor in pulmonary responses to O_3 has been demonstrated previously in other species, including humans (McDonnell et al. 1993, 1995) and rats (Gunnison et al. 1992).

Because O_3 does not penetrate epithelial membranes and murine pulmonary mast cells are located beneath the epithelium, murine mast cells are likely to modulate inflammatory responses secondary to an initial O_3 interaction with epithelium and macrophages. There is evidence from in vitro experiments that normal epithelial cell–mast cell interactions may have important consequences on the baseline function of mast cells (Peden et al. 1997). In these studies, investigators found that tissue culture medium conditioned by nonstimulated epithelial cells inhibits immunoglobulin E- and ionophore-induced mast-cell degranulation, although the inhibitory factor(s) have not been identified. It is not known whether factors released by O_3-exposed epithelial cells or macrophages could induce mast cell degranulation and secretion. It has been hypothesized that a cascade of events initiated by the interaction of O_3 with epithelial cell and macrophage membranes leads to the release of a number of signal molecules such as lipid ozonation products; these may act directly or indirectly to cause the release of other activating mediators (Pryor et al. 1995). These secondary signals may, in turn, induce mast cells to release a number of preformed and de novo–synthesized mediators that can lead to inflammatory cell infiltration and epithelial damage. Mast cells are an important source of proinflammatory cytokines, which may contribute significantly to inflammatory processes (Galli and Costa 1995). These include the pleiotropic TNF-α (Gordon and Galli 1990), which may have an important role in the pathogenesis of O_3-induced inflammation (Kleeberger et al. 1997). Mast cells also contain a number of proteases that may contribute to O_3-induced epithelial damage.

It is also important to note that the BMT procedure utilized in these studies is nonselective in terms of cell type. In addition to mast cell repletion in peripheral tissues, red blood cells were also restored in Kit^W/Kit^{W-v}-BMT mice, repairing the anemia characteristically observed in Kit^W/Kit^{W-v} mice. It may be argued that in addition to mast cells, other cells of +/+ origin contribute to the inflammatory response and epithelial cell proliferation induced by chronic O_3 exposures. To our knowledge, there is no demonstrated role of red blood cells in modulating oxidant inflammation and epithelial cell injury in the lungs. Nevertheless, the study design does not rule out the potential contribution of hemopoietic cells to the pathogenesis of O_3-induced lung effects. There was also a difference in the numbers of white blood cells between +/+ and Kit^W/Kit^{W-v} mice, and this difference was repaired by BMT. While numbers of white blood cells were lower in Kit^W/Kit^{W-v} mice, they were within the range of numbers normally found in inbred strains of mice (Russell and Bernstein 1968). Furthermore, other studies have demonstrated that leukocyte infiltration in non–mast cell mediated inflammation models is the same in Kit^W/Kit^{W-v} mice and their +/+ littermates (Galli and Hammel 1984). Therefore, it is unlikely that differences in numbers of white blood cells between the two genotypic groups account for their differential responses to chronic O_3 exposure.

Epithelial Cell Proliferation

The epithelial cell proliferative response to O_3 exposure in mast cell–deficient Kit^W/Kit^{W-v} mice was considerably smaller than the response in +/+ mice. Relative to the rate of DNA synthesis in air-exposed control animals, DNA synthesis in lung and nasal airways increased only after 14 days of O_3 exposure. At all other exposure durations, and after 35-day recovery, there were no differences between the O_3- and air-exposed groups. In O_3-exposed Kit^W/Kit^{W-v}-BMT mice, DNA synthesis increased after 3 days of exposure relative to the rate in air-exposed controls,

and synthesis rates remained elevated throughout the remainder of O_3 exposure. The time course of the proliferative response in the Kit^W/Kit^{W-v}-BMT mice was largely intermediate between those of the +/+ and Kit^W/Kit^{W-v} animals. However, like the +/+ mice, KitW/KitW-v-BMT mice showed epithelial cell proliferation in the centriacinar region of the lungs and in the nasal airways that did not return to baseline after a 35-day recovery period. Surprisingly, the number of BrdU-labeled cells actually increased significantly after the mice were removed from the O_3-exposure chamber. We conclude that, as in the inflammatory cell response, mast cells contribute to the initiation and maintenance of epithelial cell proliferation in the lungs and noses of mice after chronic O_3 exposure. It is not certain whether this effect was mediated directly by mast cells or indirectly by infiltrating inflammatory cells. Because the proliferating epithelium may have different phenotypic properties than normal, nonproliferating epithelium, it may also have different susceptibility to exogenous stimuli. It would be of interest to characterize the functional properties of the centriacinar epithelium in conditions of nonproliferation and O_3-induced proliferation.

Ventilatory Function

The ventilatory responses of Kit^W/Kit^{W-v} mice to room air following 1 day of O_3 exposure were characterized by rapid breathing (that is, increased *f*) and increased $\dot{V}_E$. In previous studies, we found a similar ventilatory response in C57BL/6J mice after 72 of hours of exposure to 0.30 ppm O_3 (Paquette et al. 1994; Tankersley and Kleeberger 1994). Although the mechanisms of this effect are not clear, we speculate that inflammation and pulmonary edema led to impeded gas exchange in the lungs, which in turn led to increased dead space ventilation. Therefore, the mice compensated by increasing $\dot{V}_E$. It is also possible that O3 exposure stimulated irritant receptors in airways and caused reflex hyperventilation. Interestingly, ventilatory responses in Kit^W/Kit^{W-v}-BMT mice were not different from those in Kit^W/Kit^{W-v} mice, but both were different from the responses in +/+ mice. These results may suggest that mast cells per se do not contribute to the differences in ventilatory responses between the Kit^W/Kit^{W-v} and +/+ mice, but that other differences between the genotypes influence the ventilatory effect of O_3 exposure.

SUMMARY AND CONCLUSIONS

These studies have demonstrated that chronic exposure to an environmentally relevant concentration of O_3 (that is, 0.26 ppm) induced significant inflammation and epithelial cell sloughing and proliferation in the lungs of mast cell–sufficient $WBB6F_1$-+/+ mice. Nasal epithelial cell proliferation was also detected, but to a much lesser degree than that observed in the lungs. The cellular responses to O_3 exposure were partially and selectively adapted over time; macrophage and PMN infiltration and epithelial cell sloughing were still significantly elevated relative to cell numbers in controls after 90 days of exposure. Furthermore, even after a 35-day recovery period from 90 days of exposure, bronchiolar epithelial cell proliferation remained significantly elevated. Ventilatory responses to O_3 exposure occurred primarily after 1 day of exposure, and ventilatory function returned to control levels thereafter. There was no apparent relation between changes in ventilation and bronchiolar inflammation, and epithelial cell responses.

The inflammatory and epithelial cell responses to O_3 exposure were significantly reduced in mast cell–deficient Kit^W/Kit^{W-v} mice with the exception of lymphocytes and total protein concentration, which were not different from values in mast cell–sufficient mice. Transplantation of bone marrow cells from mast cell–sufficient +/+ donors to mast cell–deficient Kit^W/Kit^{W-v} mice restored peripheral tissue mast cell density, and the responses to O_3 exposure in Kit^W/Kit^{W-v}-BMT mice were not different from the responses in +/+ mice. These results are consistent with the hypothesis that mast cells contribute significantly to the initiation and maintenance of bronchiolar inflammation and epithelial cell responses induced by chronic O_3 exposure in this mouse model.

A potentially important implication of these studies is that individuals with increased densities of bronchiolar mast cells may be at increased risk for O_3-induced effects. This may account, in part, for the observation that O_3-induced inflammation is greater in persons with allergic asthma compared with O_3-induced effects in healthy human subjects.

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ABBREVIATIONS

ANOVA	analysis of variance (1-way, 3-way)
BAL	bronchoalveolar lavage
BMT	bone marrow transplantion
BrdU	bromodeoxyuridine, a thymidine analog
CO_2	carbon dioxide
C × T	concentration times time
f	frequency of breathing
FEF_{25-75}	forced expiratory flow over span of 0.25–0.75 second
HBSS	Hanks' balanced salt solution
HEPA	high-efficiency particulate-filtered air
Hz	Hertz
ln	natural logarithm
LSD	least significant difference
MANOVA	multiple analyses of variance
MCD-mice	mast cell–deficient mice
MCR-mice	mast cell–replete mice
MCS-mice	mast cell–sufficient mice
N_2	nitrogen
O_2	oxygen
O_3	ozone
^{18}O	isotope of oxygen
PMNs	polymorphonuclear leukocytes (granulocyte types)
ppm	parts per million
SEM	standard error of the mean
TNF-α	tumor necrosis factor–alpha
V_T	tidal volume
$\dot{V}_E$	minute ventilation
$WBB6F_1$-Kit^W/Kit^{W-v} or Kit^W/Kit^{W-v}	mast cell–deficient mice (mouse genotype)
$WBB6F_1$-+/+ or +/+	mast cell–sufficient mice (wild type) (mouse genotype)
WBCs	white blood cells

APPENDIX: Statistical Analyses

Table A.1. ANOVA Results for Effects of Ozone or Air Exposure on the Number of Macrophages in BAL Fluid from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	660.64	330.32	7.63	0.0007
Exposure (O_3 or air)	1	6535.56	6535.56	151.02	0.0001
Time duration (days)	5	4552.54	910.51	21.04	0.0001
Genotype × exposure	2	915.97	457.99	10.58	0.0001
Genotype × time duration	10	387.78	38.78	0.90	0.5386
Exposure × time duration	5	1275.41	255.08	5.89	0.0001
Genotype × exposure × time duration	10	400.62	40.06	0.93	0.5115
Residual	144	6231.70	43.28		

Table A.2. ANOVA Results for Effects of Ozone or Air Exposure on the Number of Lymphocytes in BAL Fluid from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	1.02	0.51	4.92	0.0085
Exposure (O_3 or air)	1	4.73	4.73	45.73	0.0001
Time duration (days)	5	5.47	1.09	10.57	0.0001
Genotype × exposure	2	0.13	0.07	0.64	0.5296
Genotype × time duration	10	2.04	0.20	1.97	0.0407
Exposure × time duration	5	1.98	0.40	3.82	0.0028
Genotype × exposure × time duration	10	1.18	0.12	1.14	0.3351
Residual	144	14.91	0.10		

Table A.3. ANOVA Results for Effects of Ozone or Air Exposure on the Number of Polymorphonuclear Leukocytes in BAL Fluid from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	3.67	1.84	6.67	0.0018
Exposure (O_3 or air)	1	18.93	18.93	68.80	0.0001
Time duration (days)	5	5.86	1.17	4.26	0.0013
Genotype × exposure	2	1.94	0.97	3.52	0.0326
Genotype × time duration	10	2.04	0.20	0.74	0.6831
Exposure × time duration	5	9.34	1.87	6.79	0.0001
Genotype × exposure × time duration	10	3.29	0.33	1.20	0.3008
Residual	144	33.84	0.28		

Table A.4. ANOVA Results for Effects of Ozone or Air Exposure on the Number of Epithelial Cells in BAL Fluid from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	660.64	330.32	7.63	0.0007
Exposure (O_3 or air)	1	6535.56	6535.56	151.02	0.0001
Time duration (days)	5	4552.54	910.51	21.04	0.0001
Genotype × exposure	2	915.97	457.99	10.58	0.0001
Genotype × time duration	10	387.78	38.78	0.90	0.5386
Exposure × time duration	5	1275.41	255.08	5.89	0.0001
Genotype × exposure × time duration	10	400.62	40.06	0.93	0.5115
Residual	144	6231.70	43.28		

Table A.5. ANOVA Results for Effects of Ozone or Air Exposure on the Total Protein Concentration in BAL Fluid from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	3,594	1,797	0.49	0.6142
Exposure (O_3 or air)	1	253,125	253,125	68.89	0.0001
Time duration (days)	5	154,697	30,939	8.42	0.0001
Genotype × exposure	2	3,602	1,801	0.49	0.6136
Genotype × time duration	10	33,293	3,329	0.91	0.5294
Exposure × time duration	5	139,976	27,995	7.62	0.0001
Genotype × exposure × time duration	10	59,016	5,902	1.61	0.1103
Residual	144	529,076	3,674		

Table A.6. ANOVA Results for Effects of Ozone or Air Exposure on BrdU Uptake in Nuclei of Lung Epithelial Cells from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	3.25	1.63	7.42	0.0009
Exposure (O_3 or air)	1	32.56	32.56	148.63	0.0001
Time duration (days)	4	11.14	2.78	12.71	0.0001
Genotype × exposure	2	5.19	2.59	11.84	0.0001
Genotype × time duration	8	6.21	0.78	3.54	0.0010
Exposure × time duration	4	0.31	0.08	0.36	0.8386
Genotype × exposure × time duration	8	3.35	0.42	1.91	0.0640
Residual	120	26.29	0.22		

Table A.7. ANOVA Results for Effects of Ozone or Air Exposure on BrdU Uptake in Nuclei of Nasal Epithelial Cells from +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	0.26	0.13	0.20	0.8190
Exposure (O_3 or air)	1	8.93	8.93	14.03	0.0004
Time duration (days)	4	12.86	3.21	5.05	0.0014
Genotype × exposure	2	0.19	0.10	0.15	0.8589
Genotype × time duration	8	5.18	0.65	1.02	0.4324
Exposure × time duration	4	0.26	0.07	0.10	0.9814
Genotype × exposure × time duration	8	7.32	0.92	1.44	0.1996
Residual	120	38.82	0.64		

Table A.8. ANOVA Results for Effects of Ozone or Air Exposure on Breathing Frequency of +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	1.16	0.58	4.15	0.0201
Exposure (O_3 or air)	1	0.84	0.84	6.02	0.0168
Time duration point (days)	4	0.86	0.43	3.07	0.0532
Genotype × exposure	2	0.50	0.25	1.79	0.1748
Genotype × time duration	4	0.69	0.17	1.23	0.3053
Exposure × time duration	2	1.47	0.74	5.23	0.0076
Genotype × exposure × time duration	4	4.38	1.10	7.82	0.0001
Residual	66	9.25	0.14		

Table A.9. ANOVA Results for Effects of Ozone or Air Exposure on Tidal Volume of +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Strain	2	7,541	3,770	1.44	0.2434
Exposure (O_3 or air)	1	2,501	2,501	0.96	0.3313
Time point (days)	2	29,199	14,599	5.59	0.0057
Strain × exposure	2	3,905	1,953	0.75	0.4774
Strain × time	4	3,911	978	0.37	0.8261
Exposure × time	2	1,816	908	0.35	0.7076
Strain × exposure × time	4	11,599	2,900	1.11	0.3591
Residual	66	172,346	2,611		

Table A.10. ANOVA Results for Effects of Ozone or Air Exposure on Minute Ventilation of +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	120	59	0.41	0.6665
Exposure (O_3 or air)	1	511	511	3.49	0.0662
Time duration (days)	2	324	162	1.11	0.3374
Genotype × exposure	2	326	163	1.11	0.3344
Genotype × time duration	4	211	53	0.36	0.8366
Exposure × time duration	2	629	314	2.15	0.1251
Genotype × exposure × time duration	4	2,616	654	4.47	0.0030
Residual	66	9,668	146		

Table A.11. ANOVA Results for Effects of Ozone or Air Exposure on Minute Ventilation Response to Hypercapnic Hypoxia Challenge of +/+, Kit^{W}/Kit^{W-v}, and Kit^{W}/Kit^{W-v}-BMT Mice

Source of Variance	Degree of Freedom	Sum of Squares	Mean Square Error	*F* Value	*p* Value
Genotype	2	61,615	30,807	2.84	0.0659
Exposure (O_3 or air)	1	75	75	0.01	0.9339
Time duration point (days)	2	3,528	1,764	0.16	0.8505
Genotype × exposure	2	41,566	20,783	1.91	0.1557
Genotype × time duration	4	99,755	24,939	2.30	0.0684
Exposure × time duration	2	39,300	19,650	1.81	0.1719
Genotype × exposure × time duration	4	171,426	42,856	3.95	0.0062
Residual	66	717,066	10,865		

INTRODUCTION

In January 1994, Dr. Steven Kleeberger of the Johns Hopkins University School of Hygiene and Public Health submitted a preliminary application to the Health Effects Institute entitled *Mechanisms of Chronic Ozone Exposure: Role of Inflammation.* Kleeberger proposed to test the hypothesis that airway susceptibility to inflammation and tissue injury caused by prolonged, intermittent exposure to ozone is related to the magnitude of short-term tissue inflammation and that this inflammation is mediated, in part, by mast cells. Earlier, Kleeberger and colleagues (1993) reported that mast cells played a role in the lung inflammation response observed in mice exposed to a high concentration of ozone (1.75 parts ppm*) for three hours. In their application, the investigators planned to expose mice to 0.25 ppm ozone, which approaches environmentally relevant levels. The Health Research Committee requested a full application that addressed one of Kleeberger's two original specific aims, the possible relation between susceptibility to lung inflammation and mast cells, and asked him to include early and late time points for analysis. The Committee funded a two-year study that began in March 1995. The following Commentary on the Investigators' Report is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report into scientific and regulatory perspective.

SCIENTIFIC BACKGROUND

When inhaled, ozone can cause transient airway inflammation in humans and laboratory animals, typified by an influx of inflammatory cells (mainly polymorphonuclear leukocytes), into the airways (reviewed by Bates 1995; Balmes 1996). Other inflammatory cells, such as macrophages, lymphocytes, and mast cells, are also drawn to sites of inflammation and play a role in the inflammatory response (Lukacs et al. 1995). These cells can release protein and lipid mediators that promote continued inflammation. When activated, inflammatory cells produce highly reactive oxygen species that can injure epithelial cells, resulting in increased permeability of epithelial tissue. Epithelial tissue often responds to injury by increasing epithelial cell proliferation.

Kleeberger's study focused primarily on the contribution of mast cells to ozone-induced airway inflammation and injury. Mast cells, which are derived from bone marrow precursors, migrate to tissues throughout the body where they mature (Abbas et al. 1994). Many studies link mast cell activation with the onset of inflammation; mast cells are already present in tissues that undergo inflammation, and they produce mediators that stimulate the migration and growth of other inflammatory cells (reviewed by Abbas et al. 1994; Metcalfe et al. 1997; Costa et al. 1997; Galli 1993). Mammalian lungs contain mast cells closely associated with the overlying epithelium. They (and the levels of mast cell–derived inflammatory mediators) are more numerous in the airways of people with asthma than in healthy people (reviewed by Redington et al. 1995). Thus, breathing ozone may increase the risk of lung inflammation in people with asthma.

A secondary focus of this study was to evaluate the role of mast cells in altered breathing patterns observed following ozone exposure. Ozone increases breathing frequency, but tidal volume (the volume of air in an average breath) is reduced, producing rapid, shallow breathing (Lee et al. 1979; Tepper et al. 1991; Tankersley et al. 1993).

Mast cell research was stimulated by the development of mast cell–deficient mice (MCD-mice) by Kitamura and colleagues (1978). One chromosomal defect in MCD-mice prevents them from producing a factor required for mast-cell precursor maturation. Thus, the tissues of MCD-mice contain less than 1% mast cells and there are no mast cells in the lungs. These mice can be repleted with mast cells (MCR-mice) by intravenous transplantation of genetically compatible bone marrow cells from mast cell–sufficient mice (MCS-mice). By comparing the responses of the three genotypes to inflammatory stimuli, researchers have demonstrated the mast cells' role in several inflammatory processes (Wershil et al. 1988; Qureshi and Jakschik 1988; Suzuki et al. 1993; Kleeberger et al. 1993; Longphre et al. 1996).

In an earlier study, Kleeberger and coworkers (1993) reported that MCS-mice exposed to 1.75 ppm ozone for 3 hours developed inflammatory responses in their lungs. Mast cell–deficient mice had diminished inflammatory responses, and the responses in MCR-mice were comparable with those in MCS-mice. Later results from Kleeberger's laboratory indicated that the degree of epithelial cell dam-

* A list of abbreviations appears at the end of the Investigators' Report.

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Dr. Steven R. Kleeberger's two-year study, *Mechanisms of Response to Ozone Exposure: The Role of Mast Cells in Mice,* began in March 1995 and had total expenditures of $190,643. The Investigators' Report from Kleeberger and colleagues was received for review in September 1997. A revised report, received in March 1998, was accepted for publication in April 1998. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and in the Review Committee's Commentary.

age and proliferation followed a similar pattern in the three mouse genotypes exposed to 2 ppm ozone for 3 hours (Longphre et al. 1996). In their present study, the investigators exposed the three genotypes to a lower level of ozone and examined lung inflammation and injury after short-term, intermediate, and prolonged exposures.

JUSTIFICATION FOR THE STUDY

A major unanswered question concerning human exposure to ozone is whether prolonged or repeated exposures cause chronic inflammation that may lead to permanent damage of the small airways. The HEI Research Committee thought that the availability of the three mouse genotypes allowed a closer look at this question. In addition, Kleeberger's study of the cellular mechanisms of inflammation complemented HEI's longstanding program of research on the health effects of exposure to ozone.

OBJECTIVES

The investigators' major objective was to test their hypothesis that mast cells play a role in initiating and propagating airway inflammation and epithelial cell injury following exposure to ozone. A secondary objective was to determine whether mast cells and inflammation mediated by mast cells contribute to changes in breathing patterns.

TECHNICAL EVALUATION

STUDY DESIGN AND METHODS

This study had a strong and straightforward design. Kleeberger and colleagues exposed MCS-, MCD-, and MCR-mice to 0.26 ppm ozone or filtered air for 8 hours/day, for 1, 3, 14, 30, and 90 days (5 days a week for exposures longer than 3 days). They performed sham transplantations (substitution of saline for bone marrow) on MCS- and MCD-mice before exposures in order to ensure that the stress of the operation did not affect the experimental outcomes. Immediately following air or ozone exposures, Kleeberger and coworkers tested the animals' breathing frequency and tidal volume (the latter in room air and in air with an increased level of carbon dioxide) and then killed them to examine lung inflammation and airway injury.

An important component of this study was the assessment of the success of bone marrow transplantation. The investigators counted the numbers of mast cells in tissue from the trachea and mainstem bronchi of each genotype and compared mast cell densities. Full mast cell repletion requires at least 15 weeks (Kleeberger et al. 1993); therefore, they waited 16 weeks before beginning air or ozone exposures. Because MCD-mice are anemic (Galli and Kitamura 1987), the investigators also determined the effect of bone marrow transplantation on the numbers of red blood cells, hemoglobin levels, and hematocrits (the percentage of the volume of a blood sample accounted for by red blood cells).

An unexpected characteristic of the MCD-mice used in this study was their apparently low levels of white blood cells (WBCs) compared with the levels of MCS-mice. Galli and Kitamura (1987) reported that MCD-mice had essentially normal levels of circulating WBCs; however, Table 2 in the Investigators' Report indicates a statistically significant, 33% decrease in circulating WBCs in 25-week-old MCD-mice compared with MCS-mice. This is a concern because low levels of WBCs in these mice may prevent them from mounting a normal inflammatory response to a non–mast cell stimulus and may blunt their response to ozone. Kleeberger and colleagues acknowledge that the WBC count in their MCD-mice was lower than expected, but they point out that the level in 25-week-old mice is within the range of WBCs normally found in inbred strains of mice (Russell and Bernstein 1968). They also point to the report by Galli and Hammel (1984), which indicated that WBC infiltration in inflammation that was not mediated by mast cells was similar in MCD- and MCS-mice. Thus, they concluded that the lower WBC count did not affect the experimental outcomes.

The rationale for choosing inflammation and injury endpoints was logical and the methodology was state-of-the-art. Kleeberger and coworkers measured the inflammatory response by quantifying the numbers of macrophages, lymphocytes, and polymorphonuclear leukocytes in bronchoalveolar lavage (BAL) fluid. They assessed airway injury by measuring the protein content in BAL fluid (an indicator of epithelial tissue permeability), the number of epithelial cells released from epithelial tissue into BAL fluid, and epithelial cell proliferation in lung and nasal tissue. In separate exposures, mice of each genotype breathed ozone for 90 days and all analyses were repeated after a 35-day recovery in clean air to determine whether ozone-induced changes reversed.

The experiments assessing the role of mast cells on ozone-induced changes in breathing patterns contributed little important information to this study. The method used to measure tidal volume is indirect and depends on monitoring and estimating a number of experimental conditions. In addition, measurements of tidal volume are less accurate during bronchoconstriction (a characteristic of ozone

Table 1. Inflammation and Injury in the Airways of Mast Cell–Sufficient (MCS), Mast Cell–Deficient (MCD), and Mast Cell–Repleted (MCR) Mice Exposed to Ozone[a]

	Days on Which an Elevation of Cells in BAL Fluid[b] Was Observed		
Endpoint	MCS Mice	MCD Mice	MCR Mice
Lung Inflammation			
Macrophages	1, 3, 14, **30, 90**[e]	14, **30**[e]	1, **3**, 14, 30, **90**[e]
Lymphocytes	14	14	**14**[e], 30
PMNs	**3**[e], 14, 30	None[f]	**3**[e], 14, 30
Lung and Nasal Injury			
Epithelial Cells in BAL Fluid	1, **3**[e], 14, 30, 90	**3**[e], 14	1, **3**[e], 14, 30, 90
Epithelial Tissue Permeability (Protein in BAL fluid)	1, **3**[e], 30	1	1, **3**[e], 30
Proliferation of Lung Epithelial Cells[c]	3, **14**[e], 30, 90, 35R[d]	14	3, 14, 30, **90**[e], 35R[d]
Proliferation of Nasal Epithelial Cells[c]	3, **35R**[d]	14	3, **35R**[d]

[a] Mice were exposed to filtered air (controls) or to 0.26 ppm ozone for 1, 3, 14, 30, or 90 days. The table indicates the days on which statistically significant increases occurred in ozone-exposed mice of each genotype compared with their controls.

[b] Bronchoalveolar lavage (BAL) fluid.

[c] Epithelial cell proliferation cannot be detected following a single 8-hour exposure; therefore measurements were not made on day 1.

[d] 35R refers to a separate set of mice exposed for 90 days to ozone and allowed to recover in clean air for 35 days.

[e] Bold numbers indicate peak responses.

[f] None = no significant changes.

inhalation). The authors acknowledge the limitations of this method, but maintain that it was suitable for their purposes because their intent was to compare relative, rather than absolute, changes in tidal volume among the three genotypes.

STATISTICAL METHODS

The study design involved independent and dependent variables. The independent variables were genotype, exposure (filtered air or 0.26 ppm ozone), and time. The dependent variables were indicators of inflammation and injury and ventilatory responses. The effects of genotype, exposure, and time on the dependent variables were analyzed by three-way analysis of variance (ANOVA). The effects of bone marrow transplantation on tissue mast cell densities and blood parameters were assessed by 1-factor ANOVA. ANOVA is an appropriate method of analysis; however, given the large number of outcome variables, it would be useful to know whether the outcomes were correlated, and, if so, in what ways. The investigators could have used multiple analyses of variance (MANOVA) for a preliminary exploration of how responses to the various endpoints were related to each other. They chose not to perform MANOVA because they thought these analyses would increase the complexity of their report and would not contribute substantially to understanding the pathogenesis of injury or the manner in which mast cells contribute to the injury. The investigators present many *p*-values in their statistical analyses. Interpretation of large numbers of *p*-values can be complicated by the fact that approximately 1 of 20 *p*-values may indicate statistical significance when there is no real difference.

RESULTS AND INTERPRETATIONS

The investigators compared the inflammatory and injurious responses of the mouse genotypes in three ways: determining the days on which ozone exposure caused statistically significant elevations in inflammatory or injury endpoints in each genotype compared with their air-breathing controls (see Table 1 of this Commentary), comparing the responses of MCS- and MCR-mice, and comparing the responses of MCS- and MCR-mice with MCD-mice. The key results are discussed briefly in the following text.

Lung Inflammation

Macrophages were the cell type most dramatically affected by ozone. Macrophage numbers were elevated in the BAL fluid from MCS- and MCR-mice throughout the 90-day exposure period. Unlike other inflammatory cells, the macrophage influx in animals with normal mast cell levels did not attenuate during ozone exposure. In contrast, MCD-mice had elevated numbers of macrophages only after 14 and 30 days of exposure, and fewer macrophages were recovered from MCD-mice than from

MCS- and MCR-mice (which had similar numbers of BAL fluid macrophages). The numbers of macrophages recovered from BAL fluid returned to control levels in each genotype after a 35-day recovery in clean air.

The numbers of lymphocytes in BAL fluid were not elevated in any mouse genotype until the fourteenth day of ozone exposure. Lymphocyte levels in MCS- and MCD-mice did not differ significantly at any time point; however, lymphocytes remained elevated in BAL fluid from MCR-mice after 30 days of exposure to ozone, while levels in MCD-mice returned to control levels.

The appearance of elevated numbers of polymorphonuclear leukocytes in MCS- and MCR-mice during ozone exposure was slightly delayed compared with macrophages; this elevation was attenuated by 90 days in both genotypes. The numbers of polymorphonuclear leukocytes, however, were not elevated at any time in MCD-mice. The authors state that the mean numbers of these cells were similar in BAL fluid from MCS- and MCR-mice, and this appears to be true at most time points. However, after a 3-day exposure to ozone, the investigators recovered more polymorphonuclear leukocytes from MCS-mice than from MCR ones. This suggests that the number of mast cells in the MCR-mice at this time was insufficient to elicit a typical response to ozone, or that other cells (which were not repleted) are involved.

In summary, there was a greater influx of macrophages and polymorphonuclear leukocytes in the BAL fluid of MCS-mice exposed to ozone than there was in the BAL fluid of MCD-mice. These differences were, in general, lost when MCD-mice were repleted with mast cell precursors.

Airway Injury

The investigators reported that the number of epithelial cells recovered in BAL fluid from MCS- and MCR-mice was elevated throughout the ozone exposure period and returned to control levels after a 35-day recovery in clean air. The time course of the epithelial cell response was shorter in MCD-mice, and this genotype had fewer epithelial cells in its BAL fluid than MCS- and MCR-mice (which had similar levels).

Epithelial tissue permeability increased at three time points in MCS- and MCR-mice but only after the first day of exposure in MCD-mice. Because there were no statistically significant differences in the levels of BAL fluid protein among the genotypes at any time, the authors conclude that the effect of ozone on permeability was independent of mast cells.

Epithelial cell proliferation increased in the centriacinar region of the lungs of MCS- and MCR-mice during the exposure period and persisted after a 35-day recovery in clean air. In contrast, proliferation increased at only one time point in MCD-mice. (Cell proliferation cannot be detected after a single 8-hour ozone exposure; therefore, the first measurement was made on day 3 of exposure.) The proliferative response of nasal epithelial cells from each genotype was weaker than the proliferative response in the lung.

In summary, ozone exposure elevated three indicators of epithelial cell injury in MCS-mice: the appearance of epithelial cells in BAL fluid, epithelial cell proliferation, and epithelial tissue permeability. The duration of these responses was shorter in MCD-mice, and these responses returned to normal in MCR-mice. Because epithelial cell proliferation remained elevated in MCS- and MCR-mice after a 35-day recovery in clean air and inflammatory cells returned to control levels, it may be likely (as the investigators suggest) that signals for epithelial cell proliferation are distinct from those for inflammatory cell recruitment. Also, the observation of a persistent increase in epithelial cell proliferation after ozone exposure ceased suggests the possibility of permanent, ongoing structural changes.

There were few significant effects of ozone on breathing, and the small effects observed after one day of exposure rapidly attenuated. The similar responses of MCD- and MCR-mice indicates that mast cell repletion did not modify these effects. No association between mast cells and control of breathing has been described; therefore, the lack of mast cell involvement is not surprising.

Effects of Bone Marrow Transplantation on Tissue Mast Cells and Blood Parameters

Bone marrow transplantation restored the number of mast cells in the mainstem bronchi of MCR-mice to normal; although the number of tracheal mast cells increased, they remained lower in MCR-mice than in MCS-mice. The numbers of red blood cells, white blood cells, and platelets were significantly lower in MCD-mice compared with MCS-mice, as were the hematocrit and hemoglobin levels. With the exception of platelets (which remained depressed), bone marrow transplantation restored each parameter to levels seen in MCS-mice. As stated by the authors, these results indicate that, in addition to mast cells, bone marrow transplantation repopulates other cell types and cannot be considered selective for mast cells. They correctly conclude that their results do not rule out the possibility that other cell types may have contributed to the restoration of ozone-induced inflammation and injury in MCR-mice.

IMPLICATIONS FOR FUTURE RESEARCH

The results of Kleeberger and colleagues can be extended in several directions. First, the role of mast cell mediators in ozone-induced lung inflammation could be corroborated

by comparing their levels in mast cells in BAL fluid from MCS- and MCR-mice exposed to ozone or air. A lower content of cell-bound mediators in ozone-exposed mice might provide an important link to the inflammatory end points examined in this study. These studies could be extended to people exposed to air or ozone under controlled conditions. In addition, showing that ozone-exposed animals treated with monoclonal antibodies targeted against mast cell mediators had a lower inflammatory response than untreated animals would add further support for mast cell mediators playing a role in ozone-induced inflammation.

Second, it is important to demonstrate that there is not an inherent down-regulation of the inflammatory response in the MCD-mice. Exposing the lungs of these mice to chemokines that induce the migration of inflammatory cells (other than mast cells) to the airways would further define the ability of these mice to mount an inflammatory response not mediated by mast cells. Comparing the in vitro responsiveness of macrophages, lymphocytes, and polymorphonuclear leukocytes from MCS- and MCD-mice to exogenous stimuli could provide additional evidence that MCD-mice have normal immunological responses.

Third, because asthma is characterized by airway hyperreactivity (in addition to airway inflammation), assessing ozone-induced airway reactivity in each genotype may provide further insight into the role of mast cells in airway responses to ozone.

CONCLUSIONS

Kleeberger and colleagues confirmed and significantly extended their earlier finding that mast cells play an important role in lung inflammation and epithelial cell injury induced by exposure to ozone. In this complex and technically demanding study, they compared these responses in mast cell–sufficient, mast cell–deficient, and mast cell–repleted mice exposed to ozone from 1 to 90 days.

Exposure to 0.26 ppm ozone induced greater inflammatory response and epithelial cell injury in the lungs of mice with normal levels of mast cells than in the lungs of mast cell–deficient mice. Bone marrow transplantation from mast cell–sufficient mice restored the ozone-induced responses of mast cell–deficient mice to those of the mast cell–sufficient animals. Bone marrow transplantation replenishes more than mast cells; therefore, the contribution of other cell types to the restoration of ozone-induced changes in these mice cannot be ruled out. However, the overall results support the investigators' hypothesis that mast cells contribute significantly to ozone-induced lung inflammation and epithelial cell injury. These results provide an important contribution to understanding the mechanisms of lung injury that may occur in people exposed to ozone. Furthermore, the persistence of increased epithelial cell proliferation after ozone exposure has ceased suggests the possibility of permanent, ongoing structural changes. These findings could be especially relevant for individuals with asthma who have more mast cells in their lungs than other healthy people.

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