Mast cell activation is not required for induction of airway hyperresponsiveness by ozone in mice


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Noviski, N., J. P. Brewer, W. A. Skornik, S. J. Galli, J. M. Drazen, and T. R. Martin. Mast cell activation is not required for induction of airway hyperresponsiveness by ozone in mice. J. Appl. Physiol. 86(1): 202–210, 1999.—Exposure to ambient ozone (O3) is associated with increased exacerbations of asthma. We sought to determine whether mast cell degranulation is induced by in vivo exposure to O3 in mice and whether mast cells play an essential role in the development of pulmonary pathophysiological alterations induced by O3. For this we exposed mast cell-deficient WBB6F1-kitW+/kitW− mice and the congenic normal WBB6F1 (+/+) mice to air or to 1 or 3 parts/million O3 for 4 h and studied them at different intervals from 4 to 72 h later. We found evidence of O3-induced cutaneous, as well as bronchial, mast cell degranulation. Polymorphonuclear cell influx into the pulmonary parenchyma was observed after exposure to 1 part/million O3 only in mice that possessed mast cells. Airway hyperresponsiveness to intravenous methacholine measured in vivo under pentobarbital anesthesia was observed in both kitW+/kitW− and +/+ mice after exposure to O3. Thus, although mast cells are activated in vivo by O3 and participate in O3-induced polymorphonuclear cell infiltration into the pulmonary parenchyma, they do not participate detectably in the development of O3-induced airway hyperresponsiveness in mice.

Epidemiological studies have revealed that elevated levels of pollutant ozone (O3) are followed 1 day later by an increased number of emergency room visits and hospital admissions for exacerbations of asthma (3, 54, 61, 65). O3 exposure results in acute reductions of pulmonary function and enhanced nonspecific pulmonary bronchoconstrictor responsiveness in normal and asthmatic humans (5, 11, 16, 20, 21, 34, 35, 38) and in dogs, monkeys, sheep, and guinea pigs (4, 15, 18, 19, 37, 56). The cellular pathways leading from O3 exposure to pulmonary physiological derangements may involve polymorphonuclear cells (PMN), which have been observed in bronchoalveolar lavage samples from O3-exposed humans and animals (10, 22–24, 29–31, 47, 50, 57). These cells, and the oxygen radicals they produce when activated by O3, may participate in the development of enhanced contractile responses to cholinergic agonists (25, 43, 60) and exacerbations of clinical asthma.

Previous studies have suggested that mast cells may have an important role in the development of the airway hyperresponsiveness (AHR) induced by O3 exposure. Exposure to O3 can result in an increase in the number of mast cells in the airway epithelium (28, 48, 62), bronchial mast cell degranulation (28), and release of mast cell mediators (36, 59). Studies in mice involving bronchoalveolar lavage have found that the influx of PMN into the lung after O3 exposure is mast cell dependent (33), and other studies have suggested that O3-induced infiltration of activated PMN into the lung is necessary for development of AHR (22, 50). Furthermore, extensive acute mast cell degranulation induced by intravenous anti-IgE leads to an enhancement of pulmonary responsiveness to intravenous methacholine (MCh) in mice (41). We therefore designed experiments to determine whether O3 induces mast cell degranulation and whether the mast cell mediators thus released contribute to the development of AHR, either via a direct action on airway smooth muscle or indirectly via the induction of PMN infiltration in the lung.

For this we exposed mast cell-deficient WBB6F1-kitW+/kitW− (kitW+/kitW−) mice and the congenic normal (+/+) mice to O3 or air and then performed histological evaluation of their cutaneous and pulmonary tissues to assess the extent of mast cell degranulation and the intensity of inflammatory cell influx. Before the mice were euthanized, we measured lung conductance (GL) and dynamic compliance (Cdyn) after O3 or air exposure and also monitored changes in those parameters induced by the administration of increasing doses of intravenous MCh in vivo. We found that O3 exposure leads to cutaneous and bronchial mast cell degranulation, which in turn appears to contribute to the development of PMN infiltration into the lung parenchyma. However, neither mast cells nor PMN influx into the lung was essential for the development of O3-induced AHR.

**METHODS**

Animals. Eight- to twelve-week-old male WBB6F1+/+ (normal) and kitW+/kitW− mice were obtained from Dr. Warren Frost (Bozeman, MT). KitW+/kitW− mice possess <0.5% of the...
normal numbers of cutaneous mast cells and are completely deficient in mast cells in all other organs (27, 12) due to a mutation affecting the c-kit tyrosine kinase receptor gene (6, 14, 66). The mutant mice exhibit several other phenotypic abnormalities, including anemia, a lack of cutaneous melanocytes, and sterility, but no abnormalities of any nucleated bone marrow-derived cell type have been identified (12, 27).

Ozone exposure. Mice were exposed for 4 h to either filtered air, 1 part/million (ppm) of O₃, a level of exposure previously shown to induce neither pulmonary edema nor death in mice (8), or 3 ppm of O₃. O₃ was generated by passing a constant flow of filtered, dry 100% oxygen through a high-voltage (7,000-V) discharge-device ultraviolet source and mixing it with a diluting flow of filtered room air (50 l/min) in a stainless steel and Plexiglas exposure chamber (100 liters) maintained at a net negative static pressure of 0.5 in. of H₂O. Samples of the exposure atmosphere were continuously drawn from the exposure chamber via a sampling port at the level of the mice, and the O₃ concentration was measured continuously throughout the exposure with an O₃ chemiluminescent analyzer (model 49, ThermoElectron, Hopkinton, MA) (49). The O₃ analyzer was calibrated by reference to an ultraviolet photometer (model 49, ThermoElectron, Hopkinton, MA) (49).

Airway responsiveness measurements. Acetyl-β-methylcholine chloride (MCh; Sigma Chemical, St. Louis, MO) was dissolved in normal saline and administered through a Silastic catheter placed in a jugular vein. A starting dose of 3.3 μg/kg was infused, with subsequent doses of 10, 33, 100, 330, 1,000, and 3,300 μg/kg administered; each dose was infused in a volume of 1 μl of normal saline/kg of body weight. Maximally reduced Gl and Cdyn values after each MCh dose were expressed as percentages of their values obtained just before the infusion of that dose of MCh. Intervals of 3-5 min were allowed to elapse between doses to allow Gl and Cdyn to return to within 10% of the baseline value obtained before the preceding dose. Our O₃ exposure and physiological testing protocols were approved by the institutional Animal Care and Use Committees.

Histological studies. After completion of the physiological studies, each mouse was killed by cervical dislocation and its bronchial, cutaneous, gastric, and splenic tissues were fixed in 2.0% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.3, and stored in 2.0% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.3, until processing into 1-µm-thick, Epon-embedded, Giemsa-stained sections (9). Tissues were examined by light microscopy for determination of mast cell numbers and assessment of the extent of mast cell degranulation (64). One complete mainstem or lobar bronchial cross section per mouse was evaluated. Six ×1,000 fields of view were recorded from recordings of plethysmograph pressure detected by a pressure transducer (Celesco, Canoga Park, CA) connecting the plethysmograph chamber to a reference chamber; flow was obtained by electronic differentiation of the volume signal. Transpulmonary pressure was recorded by using a second pressure transducer (Celesco) connected between the proximal end of the tracheostomy tube and the plethysmograph. Gl and Cdyn were calculated from the recordings of volume, flow, and pressure by using standard techniques (63). The resistance of the tracheostomy tube was subtracted from the calculated total resistance, and the inverse of that difference was taken as Gl.

Fig. 1. Histologically determined numbers of normal (solid bars) and degranulated (striped bars) mast cells in 1 bronchial cross section/mouse from WBB6F₁ normal (+/+) mice euthanized at different intervals after exposure to air, 1 part/million (ppm) O₃, or 3 ppm O₃ (n = 3–11 mice/group). Error bars, 1 SE for total no. of cells. *No. of cells is significantly less than those observed in air-exposed mice at same interval, P < 0.01.
forestomach wall and six $\times 1,000$ fields of splenic capsule were assessed from each of three $+/+$ mice exposed to air or 3 ppm O$_3$ 4 h, 1 day, 2 days, or 3 days earlier. Mast cells were scored as normal (10% of cytoplasmic granules exhibiting fusion, staining alterations, or extrusion from the cell) or degranulated (>10% of granules altered as above). For assessment of pulmonary PMN infiltration, the numbers of PMN visible in six $\times 1,000$ fields of lung parenchyma were counted in the Giemsa-stained sections. Histological analyses were performed on coded slides by an investigator who was unaware of the previous treatment of the mice from which the tissues were obtained.

Statistical analysis. Comparisons between air- and O$_3$-exposed groups of mice for numbers of mast cells or PMN and extent of tissue mast cell degranulation were assessed with Fisher's exact test. GI and Cdyn values and maximal GI and Cdyn responses to MCh from mice exposed to O$_3$ were compared with those of mice exposed to air at each interval by using two-way ANOVA with mouse type and exposure as independent variables. Because there were no statistically significant differences between the responses to 1 and 3 mg/kg MCh, values from these two doses were combined for the calculations of the mean maximal GI and Cdyn responses. P < 0.05 was regarded as significant.
RESULTS

Bronchial and cutaneous mast cell degranulation. There was no histologically evident O3-induced increase in the proportion of degranulated bronchial mast cells, but the number of bronchial mast cells was reduced in the bronchi of +/- mice exposed 1 day earlier to 3 ppm O3 (Fig. 1). This suggests that some mast cells had degranulated to such an extent that they were not histologically detectable at that time; by 3 days, bronchial mast cell numbers were not different from those of air-exposed mice. O3 concentration-dependent increases in degranulation of both ear and back skin mast cells were observed (Fig. 2) and were maximal at 1 day after O3 and resolved by 3 days after O3 exposure. Histological examination of gastric and splenic tissues from three +/- mice exposed to air or to 3 ppm O3 revealed no reduction in mast cell numbers (5.6 ± 0.8) or evidence of degranulation related to O3 exposure (>92% of mast cells appeared normal). No mast cells were detected in any tissue of any kitwt/kitwt-v mouse.

PMN numbers. PMN influx was present in pulmonary parenchymal tissues 4 h to 3 days after exposure to 1 or 3 ppm O3 in +/- mice (Fig. 3). Only at 4 h after exposure to 1 ppm O3 was modest PMN infiltration observed in lungs of kitwt/kitwt-v mice. PMN influx was observed at 4 h, 2 days, and 3 days after exposure to 3 ppm O3 in kitwt/kitwt-v mice, although to a lesser extent than that seen in +/- mice.

Lung function parameters. Gil values of +/- mice exposed to O3 were mildly, but not significantly, reduced compared with those of air-exposed mice 4 h to 2 days after exposure (Table 1) and the Gil values of kitwt/kitwt-v mice were also mildly reduced at 1 day after exposure. Cdyn values of +/- mice exposed to O3 were significantly reduced compared with those in the air-exposed group at 4 h after exposure to 3 ppm O3, whereas Cdyn values of kitwt/kitwt-v mice showed no such reduction.

Cholinergic responsiveness. With the exception of Cdyn responses after 1 ppm O3 in kitwt/kitwt-v mice, maximal Gil and Cdyn responses to MCh in both WBB6F1-+/+ mice and in mast cell-deficient kitwt/kitwt-v mice exposed to 1 or 3 ppm O3 were significantly greater than those of the respective sham-exposed control groups (P < 0.001, ANOVA, Fig.4). This AHR was present by 4 h, peaked at 1 day, and persisted to 3 days after exposure. The effects of O3 on maximal responses to MCh were not significantly greater in WBB6F1-+/+ mice than in kitwt/kitwt-v mice, except for Cdyn responses after exposure to 1 ppm O3 (P < 0.0001, ANOVA).

No increase in MCh sensitivity (decrease in the smallest MCh dose to which at least a 20% decrease in Gil and Cdyn occurred) was observed in any group of O3-treated mice compared with the respective group of air-exposed mice (data not shown). Furthermore, no O3-related decrease in the dose of MCh required to induce a 50% decrease in Gil or a 20% decrease in Cdyn was observed in any group (data not shown).

DISCUSSION

We investigated the potential role of mast cells in the development of bronchopulmonary inflammation and pulmonary physiological alterations after exposure to O3. We found evidence that O3 exposure induced cutaneous and bronchial mast cell degranulation, which peaked 1 day after O3 exposure. Mast cell-deficient kitwt/kitwt-v mice, compared with wild-type +/- mice, developed little or no influx of PMN into lung parenchyma after exposure to 1 ppm O3 and much less PMN infiltration than that seen in lungs of normal mice after exposure to 3 ppm O3. These findings strongly suggest that mast cells contribute to pulmonary PMN infiltration in this setting. Although mast cells also may have contributed to the reduction in Cdyn at 4 h after exposure to 3 ppm O3, the enhancement of maximal Gil and Cdyn responses to MCh observed after exposure to O3 was mast cell independent.
Infiltration in developed in the virtual absence of pulmonary PMN contributions. Nonetheless, it remains possible that mast cells contribute, at least under some exposure conditions, via the FcεRI receptors. O3 has been shown to cause mast cell degranulation subsequent to exposure to 1 or 3 ppm O3. This effect of O3 may be related to the activation of ozonation reaction products found in tissues at body surfaces, rather than systemically.

A novel finding in the present study is the evidence of cutaneous mast cell degranulation subsequent to exposure to 1 or 3 ppm O3. This effect of O3 may be related to the recent finding of Cross et al. (7) that O3 generates biochemical effects that penetrate the skin, as indicated by the findings that lipid peroxidation product levels are increased and antioxidant levels are decreased in the cutaneous stratum corneum after O3 exposure. Our finding that the numbers and extent of degranulation of gastric and splenic mast cells were unaffected by exposure to 3 ppm O3 suggests that mast cells are degranulated only by relatively high concentrations of ozonation reaction products found in tissues at body surfaces, rather than systemically.

The mechanism whereby mast cells are activated on exposure to O3 is not well understood. O3 exposure may directly damage the mast cell membrane, leading to the release of preformed mediators from the damaged cells. Recent in vitro results (52) indicate that O3 induces the release of granule products and PGD2 from cultured mast cells only under cytotoxic conditions. Alternatively, O3 might cause noncytotoxic mast cell activation via a mechanism similar to that resulting from interaction of antigen with IgE antibody bound to the cell surface by FcεRI receptors. O3 has been shown to

### Table 1. Baseline pulmonary function values

<table>
<thead>
<tr>
<th>Exposure/Mouse Type</th>
<th>Interval</th>
<th>GL</th>
<th>Cdyn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBB6F1/+/+</td>
<td>1.15 ± 0.12</td>
<td>0.98 ± 0.12</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td>kit-kit</td>
<td>0.92 ± 0.10</td>
<td>0.97 ± 0.08</td>
<td>1.02 ± 0.11</td>
</tr>
<tr>
<td>1 ppm O3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBB6F1/+/+</td>
<td>0.93 ± 0.12</td>
<td>0.99 ± 0.20</td>
<td>1.01 ± 0.27</td>
</tr>
<tr>
<td>kit-kit</td>
<td>0.95 ± 0.05</td>
<td>0.97 ± 0.10</td>
<td>1.00 ± 0.20</td>
</tr>
<tr>
<td>3 ppm O3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBB6F1/+/+</td>
<td>0.09 ± 0.11</td>
<td>0.88 ± 0.16</td>
<td>0.87 ± 0.05</td>
</tr>
<tr>
<td>kit-kit</td>
<td>1.17 ± 0.21</td>
<td>0.80 ± 0.23</td>
<td>0.97 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>1 Day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBB6F1/+/+</td>
<td>0.027 ± 0.002</td>
<td>0.023 ± 0.004</td>
<td>0.026 ± 0.004</td>
</tr>
<tr>
<td>kit-kit</td>
<td>0.025 ± 0.001</td>
<td>0.023 ± 0.004</td>
<td>0.023 ± 0.005</td>
</tr>
<tr>
<td>1 ppm O3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBB6F1/+/+</td>
<td>0.020 ± 0.0003</td>
<td>0.022 ± 0.0005</td>
<td>0.023 ± 0.005</td>
</tr>
<tr>
<td>kit-kit</td>
<td>0.020 ± 0.0004</td>
<td>0.022 ± 0.0004</td>
<td>0.025 ± 0.006</td>
</tr>
<tr>
<td>3 ppm O3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBB6F1/+/+</td>
<td>0.019 ± 0.002*</td>
<td>0.021 ± 0.0013</td>
<td>0.021 ± 0.005</td>
</tr>
<tr>
<td>kit-kit</td>
<td>0.027 ± 0.0006</td>
<td>0.021 ± 0.0013</td>
<td>0.021 ± 0.004</td>
</tr>
</tbody>
</table>

Values are means ± SD expressed in cm H2O · ml−1 · s for pulmonary conductance (GL) and in ml/cm H2O for dynamic compliance (Cdyn). kit-kit, mast cell-deficient; +/+, normal; ppm, parts/million. *Significant difference between values for air-exposed and O3-exposed mice (P < 0.05 by Student's t-test with Bonferroni correction).
interact with other Fc receptors (53), and previous histological studies have suggested that ozone exposure induces mast cell degranulation rather than cytotoxic damage (62). Several of our observations support a noncytotoxic mechanism: 1) cutaneous mast cell numbers remained essentially stable, 2) the extent of degranulation of cutaneous mast cells returned to that seen in air-exposed mice by 3 days after O3 exposure, and 3) the numbers of histologically detectable bronchial mast cells decreased but returned to that of air-exposed mice by 3 days after exposure. Perhaps O3-induced noncytolytic degranulation of mast cells requires the participation of other cell types or neural interactions that are not present in vitro.

It is also possible that the susceptibility of mast cells to cytotoxic vs. noncytotoxic mechanisms of O3-induced mediator release varies according to the phenotype or stage of development of the mast cell. There are many well-established differences between mucosal and serosal mast cells, including their susceptibility to degranulation induced by various agents and their mediator contents (13, 51). Our observation that bronchial mast cell numbers were markedly diminished 1–2 days after exposure to 3 ppm O3 with few identifiable degranulated cells could be explained if bronchial mast cells, which are of the mucosal phenotype, tend to undergo O3-induced cytotoxic death rather than noncytotoxic degranulation.

The mast cell dependence of the O3-induced infiltration of PMN cells into the lung was revealed previously by analysis of bronchoalveolar lavage fluid (33). Our histological results suggest that the pulmonary parenchymal interstitium, rather than the bronchial epithelium, is the principal site of that infiltration. This finding differs from that of others who observed PMN influx, as well as epithelial sloughing, only in the
terminal bronchiolar regions of WBB6F1-+/+ mice (39). We are unable to explain the reason for this difference. The mediator involved in PMN infiltration may be tumor necrosis factor-α, which has been shown to be produced by activated mast cells (17) and was recently implicated in O3-induced lung injury by linkage analysis (32).

Whether asthmatic subjects are especially prone to develop bronchoconstriction or increased AHR on exposure to O3 remains controversial (20, 34, 35, 38). However, it seems indisputable, on the basis of several epidemiological studies from different countries, that increased levels of ambient pollutant O3 are associated with an increase in emergency visits and hospitalizations for asthma (54, 61, 65). Interestingly, at least two of those studies (54, 65) have revealed that the peak of such additional respiratory symptoms occurs 1 day after high ambient O3 concentrations were present, an interval that matches what we observed between O3 exposure and peak cutaneous mast cell degranulation and maximal airway responses to MCh. Although this finding establishes an interesting parallel between the natural history of O3-induced airway hyperresponsiveness in mice and in subjects with asthma, it should be noted that there may be species differences in the mechanisms of O3-induced airway hyperresponsiveness between mice and humans. Thus our study indicates that neither mast cell degranulation nor PMN influx is essential for development of pulmonary physiological alterations induced by O3 in mice, but our experiments in mice do not rule out an important role for either or both of these cell types in the development of O3-induced exacerbations of human asthma.

Additional studies will be required to identify the cellular pathways by which O3 can induce mast cell- and PMN-independent enhancement of pulmonary responsiveness to cholinergic stimulation. O3 can damage airway epithelial cells, perhaps without involving mast cells or PMN, with a time course that parallels that of O3-induced AHR (57). This damage may lead to decreases in available neutral endopeptidase, resulting in impaired degradation of the neurally derived bronchoconstrictor, substance P. Multiple mediators derived from cell types other than mast cells or PMN, including neurons, eosinophils, macrophages, and/or platelets, have been implicated in the production of O3-induced AHR, including tachykinins such as substance P (4), leukotrienes (26, 36, 44), and thromboxane A2 (36).

In summary, we found that O3 exposure led to degranulation of cutaneous and bronchial mast cells and that O3-induced influx of PMN into the lung parenchyma was reduced in mice lacking mast cells compared with normal mice. Nonetheless, AHR developed after O3 exposure even in mice devoid of mast cells. It thus appears that, although multiple cell types may participate in the pathogenesis of AHR after O3 exposure, mast cells and PMN influx were not required for its development; perhaps no single inflammatory cell type is essential for the development of O3-induced AHR.

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