Airway responsiveness after acute exposure to urban particulate matter 1648 in a DO11.10 murine model

Amy J. Archer, Jennifer L. H. Cramton, Jean C. Pfau, Giuseppe Colasurdo, and Andrij Holian. Airway responsiveness after acute exposure to urban particulate matter 1648 in a DO11.10 murine model. Am J Physiol Lung Cell Mol Physiol 286: L337–L343, 2004; 10.1152/ajplung.00202.2003.—Enhanced airway responsiveness (AR) is a well-established characteristic of asthma that epidemiological evidence has linked with inhalation of ambient particulate matter (PM). To determine whether acute exposure to urban particulate matter PM1648 can exacerbate airway responsiveness and alter the early inflammatory state, a unique murine model was created using DO11.10 mice, transgenic for a T cell receptor recognizing ovalbumin 323–339. Because these mice are sensitive to ovalbumin, immunization procedures involving adjuvant or long aerosolization procedures are not necessary and, therefore, allow for the study of an acute AR response to particulate and antigen in young animals. AR was assessed by barometric whole body plethysmography and measured by enhanced pause (Penh). PM1648 and ovalbumin were administered intranasally 72 and 4 h before to AR assessment, respectively. A dose-response relationship between PM1648 and Penh was determined, and doses at or above 500 μg had Penh values significantly higher than saline controls. Penh values of control particle titanium dioxide (TiO2) were similar to saline controls demonstrating no nonspecific particulate effect on AR. Lung lavage at time of AR assessment showed no significant inflammation due to particulate exposure or ovalbumin alone; however, PM1648/ovalbumin and TiO2/ovalbumin combinations resulted in significant neutrophilia. In addition, treatment with polymyxin B to remove surface-bound endotoxin did not significantly affect Penh levels. These results indicate that PM1648 specifically increases AR in a dose-dependent manner and that this exacerbation is not a direct response to increased neutrophil concentration, particle-bound endotoxin or nonspecific particle effects.

whole body plethysmography; ovalbumin; enhanced pause; neutrophilia

ENHANCED AIRWAY RESPONSIVENESS (AR) is a well-established characteristic of asthma that epidemiological evidence suggests may be linked with inhalation of ambient particulate matter (PM) (1, 2, 45, 51, 60, 61). Ambient air particulate has been associated with increased asthma severity (19, 25, 34, 37, 40, 41) and has been linked to increases in mortality (41–43, 50, 56), hospital admissions (5, 17, 44), respiratory symptoms, and decreases in lung function in both adults and children (11, 41, 44, 49, 55).

Individuals with airway hyperresponsiveness, a characteristic of asthma, have been identified as a population particularly susceptible to the effect of ambient air pollutants. These pollutants are nonallergic stimuli that increase airway reactivity (40). Physiological and toxicological considerations suggest that exposure to fine particles (<2.5 μm in diameter) pose significant health risks as their size is such that they can be inspired most deeply into lungs (31, 41). A comprehensive review of epidemiological data regarding fine particulate air pollution (PM2.5) and its respiratory health risks on susceptible populations determined that increased mortality occurred concurrently or within 1–5 days following an increase in air pollution (41). A study of six eastern U. S. metropolitan areas also reported that exposure to ambient fine particles was specifically responsible for observed associations with daily mortality (50). Although a clear mechanism for the action of PM in asthma exacerbation has not been identified, studies with model pollutants such as residual oil fly ash suggest that PM enhances neutrophilic inflammation (14, 20, 21, 40).

Asthma is a complex disease with many confounding factors involved in induction and exacerbation, many of which are not understood. However, typical clinical manifestations of asthma include AR, lung inflammation, and airway remodeling involving many cells and cellular products (19, 29, 39, 52, 55). This study was designed to examine the effects of particulate exposure on young mice by assessing early changes in AR and inflammatory components.

To complement the array of epidemiological findings, this study focuses specifically on the hypothesis that acute exposure to urban particulate PM1648 [standard reference material (SRM 1648) PM] induces exacerbation of AR, an asthmatic component, as can be assessed with a novel murine model. Ovalbumin (Ova)-sensitive DO11.10 mice were exposed to PM1648 and subsequently to Ova via a single intranasal administration. AR was assessed with a barometric whole body plethysmograph and measured by enhanced pause (Penh), and inflammatory cell infiltration was determined by lung lavage.

MATERIALS AND METHODS

Animals. DO11.10+/+ transgenic mice are on a BALB/c background and contain an Ova-specific T cell receptor (TCR) recognizing Ova peptide 323–339. Breeder pairs were generously provided by Corixa (Hamilton, MT). We assessed TCR presence and concentration by negatively selecting for CD3+ T cells with Cell Enrichment columns (R&D Systems, Minneapolis, MN) as per the manufacturer’s instructions. Cells recovered from the columns were stained with α-CD3 antibody and KJI-26, a monoclonal antibody directed against the mouse DO11.10 TCR (Caltag Laboratories, Burlingame, CA) and assessed with a Becton Dickinson FACScalibur flow cytometer (San Jose, CA). Flow cytometric analysis found 83% CD3+ T cells to be Ova specific. All mice were used at 4 wk of age.

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Animals were housed in microisolators on a 12:12-h light-dark cycle. The mice were maintained on an Ova-free diet and given deionized water ad libitum. Euthanasia was performed by intraperitoneal injection of a lethal dose of pentobarbital sodium. All animal procedures were approved by the University of Montana Institutional Animal Care and Use Committee.

**Particulates.** SRM 1648 PM (average size 1.4 μm) was obtained from the National Institutes of Standards and Technology (Gaithersburg, MD). Particles were collected in St. Louis, MO, over a 24-mo period and combined into a single lot to ensure a standardized reference. Titanium dioxide (TiO₂), average size 0.3 μg (Fisher Scientific, Springfield, NJ), was used as a control particle to assess nonspecific particulate effects as it has been found to be relatively biologically inert (24, 26). TiO₂ was dose matched with PM1648 (500 μg) and administered intranasally as described in *Intranasal instillation of PM1648 and challenge with Ova.*

For some experiments, PM1648 particles were treated with polymyxin B sulfate salt (Sigma Chemical, St. Louis, MO) to neutralize surface-associated endotoxin (PMX1648). PM1648 particles were suspended at 1.5 mg/ml in diluted polymyxin B (150 μg/ml of sterile water) and incubated in the dark at room temperature for 20 min with vortexing before and during incubation. Vortexing of 1 ml aliquots were ultracentrifuged for 30 min at 104,000 g, then washed with 0.5 ml sterile saline, and transferred to a negatively charged centrifuge tube. Particles were centrifuged again at 104,000 g for 30 min, supernatants were removed, and the pellets were lyophilized. The tubes were then reweighed to determine the retained particle mass and the retention efficiency (72%). Extraction of PM1648 with water alone had no effect on particle mass (38); therefore, PMX1648 was instilled at 500 μg per 30-μl volume.

Endotoxin measurements of untreated (PM1648) and control particles (TiO₂, PMX1648) were performed with the Limulus Assay Gel Clot Test Kit (BioWhittaker, Walkersville, MD). Briefly, particles were serially diluted in sterile water and exposed to Limulus amoebocyte lysate as per manufacturer’s directions.

**Intranasal instillation of PM1648 and challenge with Ova.** All PM and Ova were administered intranasally and adhered to the following time format based on preliminary findings: intranasal particle at t = 0, intranasal Ova at t = 68 h, and methacholine aerosolization/AR assessment at t = 72 h. Mice were immobilized with ketamine (2.5 mg in 100 μl of sterile saline ip) and suspended in an upright position while a 30-μl aliquot of the desired solution was slowly pipetted into the nasal passage of each mouse. All particle suspensions were freshly prepared before instillation by suspension in sterile saline and dispersed by use of a Sonicator Ultrasonic Processor (Misonix, Farmingdale, NY) for 30 s. To challenge with Ova, we administered 150 μg of Ova grade III (Sigma Chemical) in 30 μl of sterile saline intranasally.

**AR measurements.** AR was assessed by barometric whole body plethysmography (Buxco Electronics, Troy, NY) as previously described (7, 8, 23, 27). Mice were unrestrained and spontaneously breathing in one of four single animal chambers while pressure differences between this chamber and a reference chamber were recorded by a barometric analysis technique. The resulting box pressure signal is caused by volume and pressure changes during the respiratory cycle of the animal from which the tidal volumes and Penh can be calculated. Penh is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and of the timing of expiration (6). Penh was used as the measure of AR in this study.

In the plethysmograph, mice were allowed to acclimate for 5 min and then exposed for 3 min to nebulized saline and subsequently to increasing concentrations (0, 3, 6, 12, 24, 50 mg/ml) of nebulized methacholine (Sigma Chemical) and finally to prednisone (DeVilbis) via ultrasonic nebulizer. After each nebulization, recordings were taken for 3 min, and Penh values measured during each 3-min sequence were averaged. Graphs were constructed of Penh values in response to increasing methacholine concentration for each dose-matched group of mice. We determined the effect of particles at each dose by taking the area under the curve (AUC) (15, 16) from the methacholine dose-response graphs. This analysis was done using the software package PRISM, version 3.0 (GraphPad, San Diego, CA). AUC results are expressed as units (x-axis multiplied by units of the y-axis) and are graphed as means ± SE.

**Inflammatory cell infiltration assessment.** For lung lavage, mice were euthanized 72 h postparticle instillation and 4 h post-Ova instillation, corresponding with the timing of AR assessment. Mice were given a lethal injection of pentobarbital sodium (5 mg ip), and lungs were lavaged with five 1.0-ml aliquots of cold PBS. Cells were counted on a Coulter Particle Counter (Beckman Coulter, Hialeah, FL). Slides were prepared by cytocentrifugation (Cytospin III; Shandon Instruments, Pittsburgh, PA) onto positively charged microscope slides (Fisher Scientific) at 1,500 rpm for 5 min and stained with Hema 3 (Fisher Scientific). The slides were air-dried and examined by light microscopy. Cell differentials were analyzed by morphological criteria of 200 cells.

**Statistical analysis.** Statistical analysis was done using the software package PRISM, version 3.0. For dose-response experiments, Penh values were graphed according to particle dose and against increasing methacholine concentrations. Mean Penh values from each dose-matched group were analyzed by two-factor ANOVA to determine the significance of each factor (particle dose and methacholine concentration) and the independence between the two factors. We determined the overall effect of particles at each PM dose by taking the AUC for each dose-matched group. The baseline for all AUC measurements was defined as the average Penh value resulting from intranasal exposure to saline (0 μg PM1648) and challenge with the control concentration of methacholine (0 mg/ml). The resulting mean AUC values were analyzed by a one-factor ANOVA followed by Dunnett’s multiple-comparison test to a single control group to determine significant differences from saline controls (0 μg). For all other AR experiments, mean Penh values were graphed according to particle type and against increasing methacholine concentrations. Resulting Penh curves from each particle-matched group were evaluated by a two-factor ANOVA to determine the significance of each factor (particle type and methacholine concentration) and the independence between the two factors. The overall effect of particle type was determined by taking the AUC for each particle-matched group (baseline as defined above), and the resulting AUC values were analyzed by a one-factor ANOVA followed by Newman-Keuls multiple-comparisons test to show significant differences compared with multiple controls. Two-tailed P values <0.05 were considered significant and were denoted with an asterisk (see Figs. 2, 4, 6, and 7). Lung lavage data were analyzed by one-factor ANOVA followed by Newman-Keuls multiple comparison to multiple controls and significance denoted as P < 0.05.

**RESULTS**

**Dose-dependent effects of PM1648 on AR.** To determine the dose-dependent effects of PM1648, we instilled animals with increasing doses (0–750 μg) of particles before sensitization with Ova and challenge with methacholine. AR was evaluated by barometric whole body plethysmography as described in MATERIALS AND METHODS. Results are shown as Penh values for each methacholine concentration (Fig. 1). Penh values increased as particle dose increased with the highest measurements shown in the group instilled with 750 μg of PM1648. Statistical analysis of dose-response Penh curves show a significant effect of PM1648 dose on the exacerbation of AR. This effect was independent of the effect of methacholine concentration, as there was no significant interaction between these two factors.
Differences over AUC values obtained from saline/saline control group. 

Statistical analysis by 2-factor ANOVA shows significant effects for methacholine concentration and particle treatment with no significant interaction between the 2 factors. 

The effect of particles at each dose is also shown as an AUC as illustrated in Fig. 2. Although there is a general upward trend of Penh over increasing particle dose, only responses from PM1648 doses of 500 μg or higher showed statistically significant increases over saline/saline controls. After these dose-response analyses, a dose of 500 μg of PM1648 was chosen for all subsequent experiments, as 500 μg was the lowest mass of PM that showed a significant increase in the particle-induced exacerbation of AR over saline controls.

These data show that exposure to PM1648 increases AR in a dose-dependent fashion.

Effects of particulate composition on AR. To determine the specificity of particulate-induced AR enhancement, we also evaluated the control particle TiO₂. Curves were constructed with the Penh values for each methacholine concentration (Fig. 3), and the cumulative effect of particulates was expressed as an AUC (Fig. 4). TiO₂ was instilled at the same 500-μg dose as PM1648. AR, as measured by Penh, was indistinguishable in animals instilled with TiO₂ compared with saline controls (Figs. 3 and 4). These data suggest specific components of urban air particulate PM1648 may be responsible for the particle-induced exacerbation of AR.

PMX1648 were evaluated to determine the role of surface-associated endotoxin on the exacerbation of AR by PM1648. AR, as measured by Penh, was similar in animals instilled with PMX1648 to those instilled with untreated particles (Figs. 5 and 6). A Limulus assay detected residual endotoxin on PMX1648 [1.04 endotoxin units (EU)/mg]; however, identical polymyxin B treatment (52) rendered endotoxin nonbioactive as determined by cytokine production of alveolar macrophages. In addition, a Limulus assay determined control particle TiO₂ also contained trace amounts (0.02 EU/mg) of endotoxin. These data suggest that although PM1648 contains endotoxin on its surface, the endotoxin does not seem to be responsible for the particle-induced exacerbation of AR.

Assessment of lung inflammation. To determine the role of inflammation on enhanced AR in this model, we lavaged lungs 72 h postparticle instillation and 4 h post-Ova instillation as described in MATERIALS AND METHODS. Whole lungs were lavaged and total cell numbers are represented in Fig. 7. Alveolar macrophage numbers were not significantly altered by exposure to PM1648 and/or Ova at the time of death as determined by statistical analysis. In this model, eosinophil influx was not noted in the lavage fluid, nor was there evidence of interstitial inflammation 72 h after PM1648 instillation compared with saline controls when determined by histological analysis (data not shown). Although there was a slight increase...
in lavaged neutrophils (PMNs) after PM1648 exposure and after Ova exposure alone, there was a statistically significant influx of PMNs after exposure to both PM1648/Ova and TiO$_2$/Ova. This suggests that either particle may prime the lung for an inflammatory response when presented with antigen, but that PM1648-induced AR enhancement may be independent of inflammatory response at the early time points examined in this study.

**DISCUSSION**

Typically, airway-responsive animal models entail rigorous immunization protocols involving adjuvant and/or long aerosolization sensitization periods (3, 18, 32, 48, 54, 58); however, these immunization and sensitization procedures may disrupt a natural Th1/Th2 balance due to the use of adjuvant (36).
Because of the necessity of multiple antigen exposures, these immunized mouse models limit the study of acute antigen/PM exposure as well as the particulate effects on young animals. To assess exacerbation of AR by acute exposure to PM1648, we developed a mouse model using DO11.10 mice transgenic for Ova peptide 323–339. Because these mice are naïvely sensitive to Ova, prior immunization and aerosolization procedures are unnecessary.

DO11.10 mice exposed to a single dose of Ova exhibit significantly higher AR, as measured by Penh, compared with saline controls. In addition, AR of unsensitized BALB/c mice (background of DO11.10 mice) 4 h after intranasal instillation of Ova was similar to saline-exposed DO11.10 mice (data not shown), verifying that the enhanced AR seen in Ova-exposed animals is a result of antigen-dependent T cell activation in the transgenic animals. These results indicate this model effectively assesses AR directly resulting from antigen exposure.

To correspond with epidemiological evidence associating acute response to elevated levels of particulate air pollution concentrations with decreases in lung function (41), we assessed AR to urban particulate matter PM1648 at 72 h post-exposure. The present study confirms that acute exposure to PM1648 significantly exacerbates AR in a dose-dependent manner at that time (Figs. 1 and 2). The dose of PM is therefore implicated in the severity of AR, thus supporting previous epidemiological findings (46). Although there is currently debate over the validity of plethysmography and Penh as a measure of AR (10, 23), this method of airflow assessment has been verified with invasive measures of lung mechanics and supported by previous findings (10, 23).

To assess a nonspecific particle effect on AR exacerbation, we evaluated Penh of TiO2-exposed animals. Unlike PM1648, TiO2 did not cause significantly increased AR above saline/Ova controls (Figs. 3 and 4), suggesting AR exacerbation may be linked to specific particle composition. Surface components of ambient PM generally consist of sulfates, nitrates (41, 50, 59), acids, and metal and carbon particles, with various chemicals adsorbed onto their surfaces (38, 41). Studies recently reviewed elsewhere have implicated soluble metals, the strong acid fraction, and/or other components of PM1648 as possible bioactive mediators of ambient PM, which may be responsible for the significant exacerbation of AR after PM and antigen exposure (38, 41). A potential explanation may be that PM could increase antigen-presenting capabilities of alveolar macrophages, thus resulting in more effective processing of the antigen, Ova, to which DO11.10 mice are sensitive. The actual role of the activated Ova-responsive T cells in AR demonstrated here remains to be elucidated.

Another surface component of PM that may influence bioactivity is the presence of ambient endotoxin. To determine the effect of surface-associated endotoxin contamination of PM1648 on the exacerbation of AR, we washed samples with polymyxin B before AR assessment. Previous studies with polymyxin B treatment of PM have shown that polymyxin B inhibits the particle-induced production of cytokines by alveolar macrophages (4, 12, 40, 52). As shown in Figs. 5 and 6, polymyxin B treatment of PM1648 had little effect on the particle-induced exacerbation of AR. Although a Limulus assay detected residual endotoxin contamination on PMX1648, identical treatment of PM eliminated cytokine production attributed to surface-associated endotoxin (52). In addition, a recent study utilizing a rat model determined that aerosolization of endotoxin in increasing concentrations did not significantly alter AR to methacholine (9), consistent with our results. Furthermore, washing PM1648 with HNO3 completely eliminated surface endotoxin, whereas Penh values of acid-washed PM1648 and untreated PM1648 were indistinguishable at equal mass doses (data not shown). These findings in conjunction with the current AR assessment suggest that surface-associated endotoxin is not a significant contributing factor in exacerbation of AR in this model.

The presence of PM and cellular infiltration in the lung at the time of AR assessment was determined by lung lavage. At 72 h postexposure, PM was present in the lung lavage fluid, lavaged alveolar macrophages, and histological tissue sections (data not shown). These observations correspond with a review (29, 30) indicating that aerosol particles deposited on the alveolar epithelium of the lungs under normal conditions are phagocytized within hours by alveolar macrophages.

Because cell profiles of the lung are an important aspect of airway hyperresponsiveness, lavage was performed at time points corresponding to AR assessment, and results are illustrated in Fig. 7. Macrophage numbers remained consistent between all particulate-, Ova-, and saline-exposed animals. However, PMN numbers were significantly increased after the instillation of both particulate (PM1648 or TiO2) and Ova, but not particulate, Ova, or saline independently. This result suggests either particle may prime the lung for an inflammatory response when sensitive individuals are presented with antigen. Interestingly, PMN infiltration was indistinguishable between animals treated with TiO2/Ova and PM1648/Ova, whereas there was a significant difference in Penh, suggesting AR exacerbation is not directly attributable to PMN influx.

Clinical studies have recently discovered unique subgroups of patients with high levels of PMNs without eosinophilia compared with control subjects (22, 33, 53), primarily in very young children. Some similar DO11.10 and BALB/c models of airway hyperresponsiveness were also not associated with elevated lung eosinophilia (3, 57, 58). A recent review provides evidence that, at most, 50% of asthma cases are attributable to eosinophilic airway inflammation, and it is believed that other mechanisms, primarily neutrophilic airway inflammation, may be involved in enhancing bronchial reactivity and reversible airflow obstruction characterizing asthma (13). This neutrophil influx, as determined by bronchoalveolar lavage of asthma patients, is potentially triggered by environmental exposures, such as particulate air pollution and ozone, and is consistent with the results in this study. However, the actual role of PMNs and/or eosinophils in this study is not clear and they may be serving as biomarkers rather than direct role players.

Although PMNs are elevated in the current model after particulate/antigen exposure, they did not appear to enhance AR directly; therefore, there may be other cellular components responsible for the particle-exacerbated AR response to antigen in this model. Mechanisms of this exacerbation may be distinguished with this model, which allows for single particle/antigen exposure and assessment of AR in very young to aged mice. This model may also be used to elucidate contributing physical characteristics of PM and biological mechanisms involved in exacerbation of AR resulting from acute exposure to urban particulates.
In summary, epidemiological evidence has shown that ambient air particulate exacerbates AR that can lead to increased morbidity and mortality. The findings support that evidence and clearly demonstrate particle-specific exacerbation of AR in a controlled environment using a unique animal model. This exacerbation results from a single acute exposure to PM and appears to be particle specific and independent of endotoxin contamination. Although preexposure to particulate matter seems to prime the lung for significant influx of PMNs, this influx alone does not directly contribute to the increase in AR.

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GRANTS

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REFERENCES


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