

Effects of Residual Oil Fly Ash (ROFA) in Mice with Chronic Allergic Pulmonary Inflammation

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ABSTRACT

Exposure to particulate matter (PM) air pollution is associated with increased asthma morbidity. Residual oil flash ash (ROFA) is rich in water-soluble transition metals, which are involved in the pathological effects of PM. The objective of this study was to investigate the effects of intranasal administration of ROFA on pulmonary inflammation, pulmonary responsiveness, and excess mucus production in a mouse model of chronic pulmonary allergic inflammation. BALB/c mice received intraperitoneal injections of ovalbumin (OVA) solution (days 1 and 14). OVA challenges were performed on days 22, 24, 26, and 28. After the challenge, mice were intranasally instilled with ROFA. After forty-eight hours, pulmonary responsiveness was performed. Mice were sacrificed, and lungs were removed for morphometric analysis. OVA-exposed mice presented eosinophilia in the bronchovascular space ($p < .001$), increased pulmonary responsiveness ($p < .001$), and epithelial remodeling ($p = .003$). ROFA instillation increased pulmonary responsiveness ($p = .004$) and decreased the area of ciliated cells in the airway epithelium ($p = .006$). The combined ROFA instillation and OVA exposure induced a further increase in values of pulmonary responsiveness ($p = .043$) and a decrease in the number of ciliated cells in the airway epithelium ($p = .017$). PM exposure results in pulmonary effects that are more intense in mice with chronic allergic pulmonary inflammation.

Keywords: asthma; experimental asthma models; ROFA; particulate matter; epithelial remodeling; eosinophils; pulmonary responsiveness.

INTRODUCTION

Several epidemiological studies have shown that elevated levels of particulate urban air pollution are associated with increases in morbidity and mortality, particularly among those with chronic respiratory and cardiovascular diseases (Fairley 1990; Pope et al. 1992; Pope et al. 1995; Schwartz 1994; Schwartz and Dockery 1992). People with asthma are one of the groups particularly affected by particulate air pollution, but the mechanisms underlying this increased sensitivity of asthmatics are not well understood. Levels of inhalable particulate matter (PM) ($< 10 \mu\text{m}$ mass median aerodynamic diameter, PM₁₀) below accepted air quality standards have been correlated with the risk of hospitalization for asthma (Slaughter et al. 2003; Yu et al. 2000).

Ambient levels of particulate air pollution trigger cardiopulmonary inflammation, with an increase in proinflammatory mediators such as IL-1, IL-6, and TNF α in the lung (Ishii et al.

2004; van Eeden et al. 2001). PM can cause an amplification of the pulmonary inflammation associated with asthma. It has been shown that some components of urban PM, such as diesel exhaust particles, can enhance allergen-induced airway inflammation and production of antigen-specific IgE and/or IgG (Dong, Yin, Ma, Millecchia, Barger et al. 2005). Conversely, it has been suggested that the lungs of susceptible people may be primed by some previous condition, such as the presence of inflammation, that enhances the effects of PM (Schildcrout et al. 2006).

The use of animal models of pulmonary allergic inflammation has provided some insight into the possible mechanisms of the worsening of asthma in the presence of PM pollutants. Studies with PM administration in mouse models of asthma sometimes resulted in apparently contradictory results concerning the exacerbation of pulmonary inflammation, probably because of different protocols of allergen sensitization and challenge and particulate composition, dosage, and administration (Dong, Yin, Ma, Millecchia, Barger et al. 2005; Dong, Yin, Ma, Millecchia, Wu et al. 2005; Goldsmith et al. 1999; Lambert et al. 2000; Steerenberg et al. 2003). There are also substantial strain differences in the modulation of allergic airway inflammation, antigen-specific IgE and/or IgG responses, and airway remodeling in mice (Ichinose et al. 2004; Shinagawa and Kojima 2003; Singh et al. 2005; Takeda et al. 2001; Whitehead et al. 2003).

Residual oil fly ash (ROFA) has been used in experimental studies as a surrogate particle to investigate the mechanisms of

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Abbreviations: BAL, bronchial alveolar lavage; CEMIB, Multidisciplinary Center for Biological Investigation, State University of Campinas; HE, hematoxylin-eosin; IgE, immunoglobulin E; IgG, immunoglobulin G; IL, interleukin; Mch, methacholine; NIH, National Institutes of Health; OVA, ovalbumin; PAS-AB, periodic acid-Schiff and alcian blue; PM, particulate matter; ROFA, residual oil fly ash; SEM, standard error mean; TNF α , tumor necrosis factor α .

the responses to PM inhalation in experimental animals (Alessandrini et al. 2006; de Haar et al. 2005; Hao et al. 2003; Kips et al. 2003). ROFA is PM collected in oil-burning power plants, and it is homogeneous and rich in water-soluble transition metals. ROFA administration to mice with allergen-induced pulmonary inflammation resulted in an increase in Th2 cytokine production, eosinophil recruitment, and airway hyperresponsiveness (Gavett, Madison, Stevens et al. 1999; Goldsmith et al. 1999; Hamada et al. 2000; Hamada et al. 1999; Lambert et al. 2000).

Our study was designed to examine the effects of intranasal administration of ROFA on pulmonary inflammation, responsiveness, and excess mucus production in a mouse model of pulmonary allergic inflammation. We used low doses of ROFA, equivalent to the amount inhaled in one day by a mouse living in the city of São Paulo.

METHODS

Study Animals

Twenty-eight BALB/c mice ($n = 7$ for each group) were obtained from CEMIB University of Campinas (Campinas, Brazil). All mice were male and six weeks old. The mice were housed in conditions of constant temperature and relative humidity and were fed a standard mice diet. They were kept free from all evidence of infectious diseases. Mice received humane care in compliance with the "Guide for Care and Use of Laboratory Animals" (NIH publication 85-23, revised 1985). The study was approved by the Institutional Review Board of the School of Medicine, University of São Paulo.

OVA Sensitization and Residual ROFA Exposure

Mice were sensitized with an intraperitoneal injection of 50 μ g of OVA (Advanced Nutrition, Rio de Janeiro, Brazil) with 6 mg aluminum hydroxide (Alum-Pepsamar, Sanofi-Sinthelabo, Rio de Janeiro, Brazil) on days 1 and 14. They were then challenged on days 22, 24, 26, and 28. Mice were placed in a plexiglass box (40 x 27 x 13 cm) coupled to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil). A solution of OVA 1% (Advanced Nutrition, Rio de Janeiro, Brazil) diluted in 0.9% NaCl (normal saline, SAL) was prepared. This solution was continuously aerosolized into the environment for thirty minutes.

Control groups received an intraperitoneal injection of 6 mg of alum and were exposed to nebulized saline (NaCl 0.9%), the vehicle of OVA, for thirty minutes.

ROFA was collected from the solid waste incinerator of the University Hospital of the University of São Paulo, which is powered by combustible oil. The element composition of ROFA was determined by neutron activation analysis (Carvalho-Oliveira et al. 2005). On days 22, 24, 26, and 28, one to three hours after nebulization of either OVA solution or saline, mice were intranasally instilled with saline or ROFA, 60 μ g of ROFA diluted in 50 μ L of saline per dose (modified from Gavett, Madison, Stevens et al. 1999).

Characterization of ROFA

Particles used in this investigation were evaluated by neutron activation to determine this elemental composition, as well as by gas chromatography and high performance liquid chromatography for organics (Nagato 2007). Presence of toxic elements, such as As, Co, Li, and Zn, and several polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, acenaphthylene, fluorene, acenaphthene, anthracene, flouranthene, phyrene, B[a]anthracene, B[k]fluorantene, B[a]pyrene, DB[ah]anthracene, B[ghi]perylene, and ind[123cd] were detected.

Lung Responsiveness

On day 30, pulmonary responsiveness of mice to increasing concentrations of aerosolized methacholine (Mch) was measured through a whole-body plethysmography system (BUXCO, Winchester, UK). Briefly, each mouse was placed in a chamber, and continuous measurement of the box pressure-time wave was made via a transducer connected to a computer data acquisition system. The main indicator of air bronchoconstriction, enhanced pause (Penh), which shows correlation with airway resistance as measured according to standard evaluation methods in BALB/C mice (Adler et al. 2004), was calculated from the box pressure-time wave form (Hamelmann et al. 1997). After measurement of baseline Penh, either aerosolized saline or Mch in increasing concentrations (6, 12, 25, 50 mg/ml) was nebulized through an inlet of the chamber for three minutes. Penh values for each dose were collected for five minutes and averaged. The area under each dose-response curve was analyzed.

Histology and Morphometry

After pulmonary responsiveness measurements, mice were anesthetized (thiopental, 33 mg) and sacrificed by aorta dissection. Lungs were removed in block and fixed in formalin 4% for forty-eight hours. After fixation, the left lung was cut longitudinally (cranial and medial lobes), and the right lung was presented transversally (cranial and medial lobes). Lung samples were imbedded in paraffin wax, and 5 μ m-thick sections were obtained for optical microscopy analyses.

Periodic acid-Schiff and alcian blue (PAS-AB) staining was performed to quantify acid and neutral mucins. In this technique, neutral and acidic glycoproteins are stained in red and blue, respectively (Jones and Reid, 1978). The quantification of neutral and acidic mucosubstances was accomplished with an optical microscope provided with an integrating eyepiece with a known area (10,000 μ m² at a magnification of 1000X) containing 50 lines with a point in both extremities. Under a magnification of 1000X, the volume fraction of neutral and acidic mucus (stored) was determined by point counting (Camargo Pires-Neto et al. 2006). The area of secretory (neutral and acidic) and ciliated cells was determined by counting the number points of the eyepiece that hit these structures, divided by the number of points corresponding to the total area of the epithelium, under a

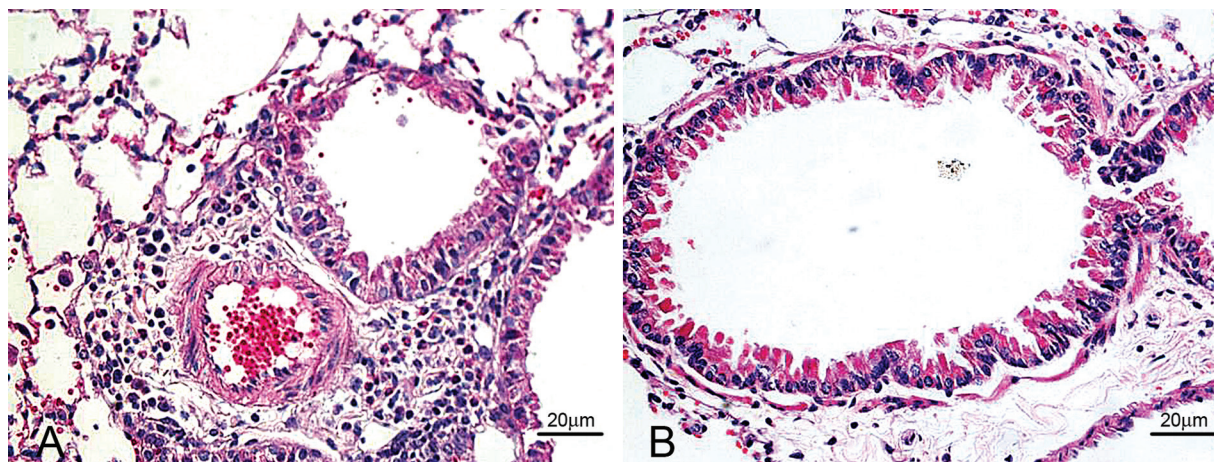


FIGURE 1.—Photomicrographs of lungs from BALB/c mice exposed to OVA (A) and to saline (B), stained with HE (magnification: 400X).

magnification of 1000X. Conventional point-counting was performed in the total length of the respiratory epithelium in each analyzed region.

Hematoxylin-eosin (HE) staining was performed to quantify eosinophils in the peribronchovascular space. The density of eosinophils was determined by counting the number of eosinophils presented in the inflammatory infiltrate between the bronchus and the adjacent artery divided by the number of points corresponding to the total area of tissue, under a magnification of 1000X. On each slide, five airways were analyzed.

Sections were deparaffinized and hydrated. After blocking endogenous peroxidase, antigen retrieval was performed with either high temperature citrate buffer (pH = 6) or trypsin. The primary antibody used was anti-mouse macrophage marker MAC-2 (1:10,000, clone M3/38; Cedarlane, Hornby, ON, Canada). The Vectastin ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used as secondary antibody. The sections were counterstained with Harris hematoxylin. For negative controls, the first antibody was omitted from the procedure, and bovine serum albumine (BSA) was used instead. The density of macrophages was determined by counting the number of macrophages presented in the inflammatory infiltrate between the bronchus and the adjacent artery divided by the number of points corresponding to the total area of tissue, under a magnification of 1000X. On each slide, five airways were analyzed.

Statistical Analysis

Parametric values are expressed as means $\pm$ SEM, and non-parametric values are expressed as medians and percentiles. Statistical analysis was performed using SigmaStat software (SPSS Inc, Chicago, IL, USA). To study parametric data, we used two-way analysis of variance followed by the Holm-Sidak method for multiple comparisons. Nonparametric data were evaluated using analysis of variance on ranks followed by Dunn's method for multiple comparisons. A *p* value of less than .05 was considered statistically significant.

RESULTS

A dense infiltration of eosinophils was present in the lung tissue between adjacent bronchi and vessels in OVA-exposed mice (Figure 1). Chronic exposure to OVA increased the amount of intraepithelial mucosubstances and epithelial thickness (Figure 2).

Lung Responsiveness

Lung responsiveness of mice to increasing concentrations of aerosolized Mch was measured using whole-body plethysmography (Figure 3). We analyzed Penh area under the dose-response curve of each group. OVA exposure induced an increase in lung responsiveness ($p < .001$). Moreover, nonsensitized mice that received ROFA also presented a substantial increase in the responsiveness ($p = .004$). Additional effects of ROFA-exposure were observed in mice exposed to OVA ($p = .04$).

Lung Inflammation

Mean values ($\pm$ SEM) of eosinophils and macrophages in peribronchovascular space are shown in Table 1. There was a significant increase in both the number of eosinophils/mm² and the number of macrophages/mm² in lung tissue in OVA-exposed mice (OVA + SAL) compared to corresponding control mice (SAL + SAL) ($p < .001$). Exposure to ROFA had no additional significant effect in eosinophil or macrophage accumulation.

Epithelium Evaluation

Exposure to OVA led to a marked increase in the thickness of the airway epithelium (Figure 4) ($p = .003$). Exposure to ROFA had no additional effect in the thickness of airway epithelium.

OVA exposure also resulted in an increase in the number of secretory cells in airway epithelium ($p < .002$) (Figure 4). No effect of ROFA was observed in mice with chronic inflammation induced by OVA.

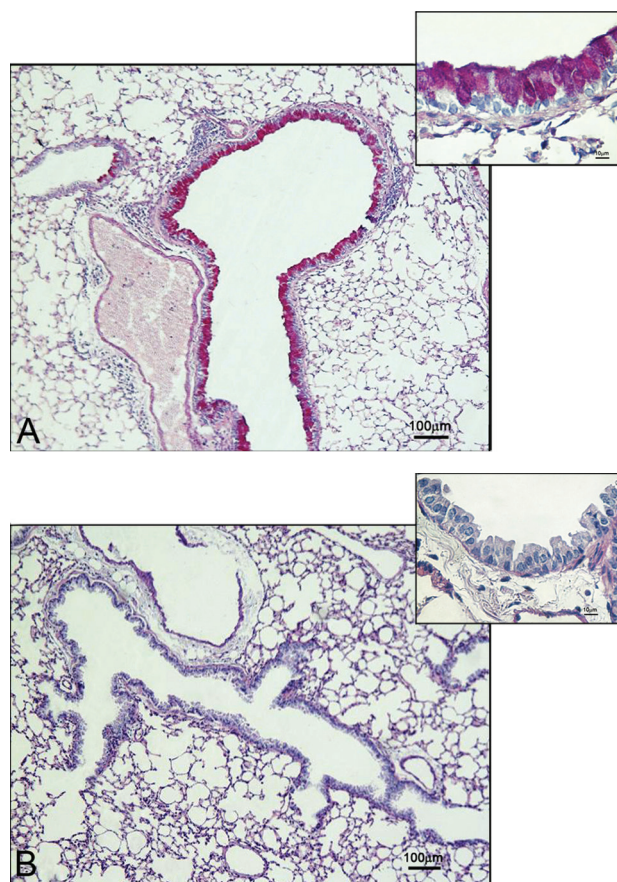


FIGURE 2.—Photomicrographs of airway epithelium from BALB/c mice exposed to OVA (A) and to saline (B), stained with periodic acid Schiff and alcian blue (magnification: 100X; inset: 1000X).

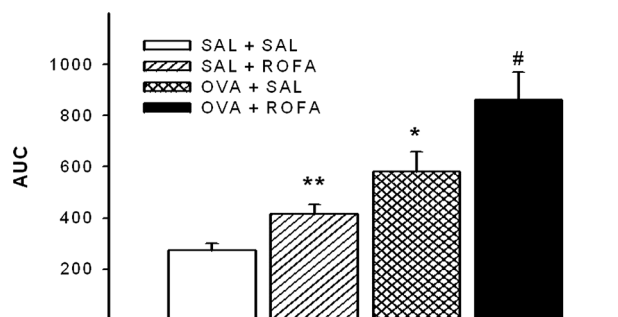


FIGURE 3.—Area under the dose-response curve to Mch (Penh). ROFA induced an increase in pulmonary responsiveness in nonsensitized mice (SAL + ROFA, $**p = .004$). Moreover, OVA sensitization (OVA + SAL, $*p < .001$) also induced an increase in responsiveness. There was an additional effect of ROFA in sensitized mice (OVA + ROFA, $\#p = .04$ compared to the other three groups) ($n = 7$ for each group).

A substantial decrease in the number of ciliated cells (Figure 4) was observed in OVA-exposed mice compared to control groups ($p < .001$). Nonsensitized mice that received ROFA also presented a substantial decrease in the area of ciliated

TABLE 1.—Inflammatory cells in peribronchovascular airway space.

Cells $\times 10^3/\text{mm}^2$	Eosinophils	Macrophages
SAL+SAL	0.24 ± 0.01	0.78 ± 0.30
SAL+ROFA	0.28 ± 0.10	0.33 ± 0.05
OVA+SAL	$1.32 \pm 0.28^*$	$2.83 \pm 0.85^*$
OVA+ROFA	$1.25 \pm 0.13^*$	$1.59 \pm 0.39^*$

Abbreviations: OVA, ovalbumin; ROFA, residual oil fly ash; SAL, saline.

* $p < .001$, compared to SAL+SAL group.

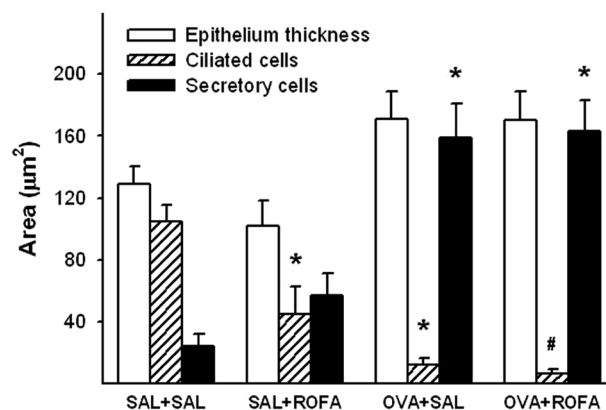


FIGURE 4.—Area of epithelium thickness and ciliated and secretory cells of airway epithelium. There was a significant increase in epithelium thickness ($*p = .003$) and in secretory cell area ($*p < .001$), and a decrease in ciliated cells ($*p < .001$) in mice chronically exposed to OVA. ROFA administration induced a decrease in ciliated cell area ($*p = .006$) in nonsensitized mice and amplified the decrease in ciliated cell area induced by OVA ($\#p = .017$ compared to the other three groups) ($n = 7$ for each group).

cells ($p = .006$). An additional effect of ROFA exposure was observed in mice exposed to OVA ($p = .017$).

The quantification of intraepithelial mucosubstances is shown in Figure 5. Volume fraction of acidic mucus was increased in all mice exposed to OVA ($p = .04$). Chronic OVA exposure resulted in an increase in the volume fraction of neutral mucus ($p < .001$). We did not observe any statistically significant effect of ROFA exposure in the amount of neutral or acidic mucus.

DISCUSSION

The use of animal asthma models has provided important insights into immune and inflammatory mechanisms of this disorder (Emala and Hirshman 1996; Herz et al., 1996; Pauwels et al., 1997). The general approach involves sensitization of mice by intraperitoneal injection of allergen (active and systemic sensitization) or local allergen exposure, usually in combination with adjuvant material such as alumen ($\text{Al}[\text{OH}]_3$). In these models, there is a characteristic eosinophilic inflammatory response that is dependent on elaboration of Th2-type cytokines (Hussain et al. 2001). After subsequent local (pulmonary) allergen challenge, these sensitized mice are evaluated for the asthmatic phenotype, and they present airway hyperresponsiveness; pulmonary

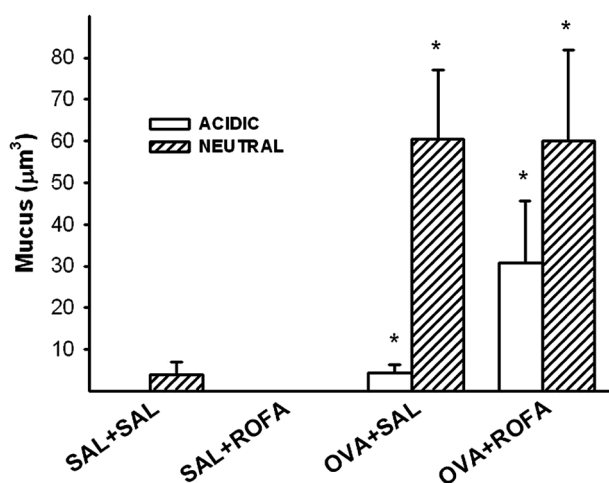


FIGURE 5.—Volume of acidic and neutral mucus stored in airway epithelium. There was an increase in the amount of neutral (* $p < .001$) and acid mucus (* $p = .036$) substances in OVA mice ($n = 7$ for each group).

inflammation; elevated serum immunoglobulin, especially antigen-specific immunoglobulin E (IgE) and IgG (Hamada et al. 1999; Ichinose et al. 2004; Singh et al. 2005; Törmänen et al. 2005); and airway remodeling, including thickened basement membrane, epithelial changes, subepithelial fibrosis, increased smooth muscle mass, and changes in airway mucosal vascularity (Tanaka et al. 2001; Törmänen et al. 2005). Törmänen et al. (2005) showed an increased proliferation of bronchial epithelial cells and peribronchial and perivascular eosinophilia induced by OVA challenges. Tanaka et al. (2001) demonstrated that prolonged antigen exposure can induce airway remodeling associated with bronchial hyperresponsiveness in sensitized mice, including collagen deposition beneath the basement membrane and goblet cell hyperplasia/hypertrophy.

Increased morbidity in persons suffering from inflammatory lung diseases such as asthma and bronchitis has been associated with air pollution particles. One hypothesis is that inhalable particles can cause an amplification of the pulmonary inflammation associated with these diseases, thus worsening the affected individual's symptoms (Goldsmith et al. 1999). Epidemiological studies have suggested that asthmatics are more sensitive than healthy persons to the effects of PM (Schildcrout et al. 2006). Although the mechanisms of PM's adverse health effects are still poorly understood, the particle's chemical composition may play a role in toxicity. ROFA and other fly ash particulates are products of oil combustion and contain soluble sulfates and transition metals. ROFA has been used as a surrogate for ambient air particle pollution, since it is rich in transition metals and its composition can be precisely determined. ROFA can cause airway epithelial injury and lung inflammation via metal-dependent oxidant injury, induction of cytokine expression (Gavett, Madison, Stevens et al. 1999) and stimulation of prostaglandin production (Carter et al. 1997; Dye et al. 1997; Gavett, Madison, Chulada et al. 1999; Samet et al. 1996). In our study, intranasal administrations of ROFA in nonsensitized mice resulted in a substantial

increase in pulmonary responsiveness and a decrease in the number of ciliated cells. However, studying the airways forty-eight hours after the last instillation of ROFA, we did not observe an increase in the number of secretory cells or mucosubstances (neutral or acidic) present in the airways.

In our study we decided to administer ROFA intranasally to simulate ambient exposure. In addition, we calculated the mean PM daily exposure in a mouse living in the city of São Paulo and administered an equivalent dose of ROFA. Our purpose was to evaluate the effect of ambient levels of PM in pulmonary inflammation, responsiveness and remodeling.

The influence of ROFA administration on experimental models of asthma has been previously studied, using different experimental protocols (Gavett, Madison, Stevens et al. 1999; Goldsmith et al. 1999; Hamada et al. 1999; Hamada et al. 2000; Lambert et al. 2000). In an experimental model using juvenile mice sensitized with OVA and coexposed to aerolized ROFA leachate, Hamada et al (1999) observed that ROFA increased airway responsiveness in mice sensitized with OVA and the number of inflammatory cells (neutrophils and eosinophils) in bronchoalveolar fluid twenty-four hours but not forty-eight hours after administration. In nonsensitized animals, no marked effect of ROFA was observed. Gavett, Madison, Stevens et al. (1999) used a single challenge of OVA in sensitized mice and administered a single intratracheal dose of ROFA one to three hours after OVA challenge and observed that ROFA potentiated the increase in eosinophils in BAL induced by OVA challenge. In addition, the increase in pulmonary responsiveness to Mch induced by OVA challenge was greater in the mice that also received ROFA. Goldsmith et al. (1999) sensitized neonate BALB/c mice to OVA and submitted them to three OVA challenges on days 21, 22, and 23 and to coexposure to ROFA aerosol on day 22. Mice that received OVA and ROFA presented higher pulmonary responsiveness to Mch but not a greater number of eosinophils in BAL compared to mice that received only OVA. In our study we evaluated eosinophil infiltrate in lung tissue forty-eight hours after the last challenge and did not observe an effect of ROFA administration on eosinophils.

Metal-rich particles have been found to enhance allergic responses to OVA and house dust mites (Campen et al. 2002; Lambert et al. 1999; Lambert et al. 2000) and to induce the increased release of allergen-related cytokines, eosinophil recruitment, and airway hyperresponsiveness in mice (Gavett, Madison, Stevens et al. 1999). Cellular reactive oxygen species generation in polymorphonuclear leukocytes was significantly correlated with insoluble silicon, iron, manganese, titanium, and cobalt, but not with soluble transition metals, and deferoxamine treatment did not affect reactive oxygen species generation (Pralhad et al. 1999).

Organic compounds also appear to play an important role in PM-induced cytotoxicity. Organics, such as PAH, have been shown to induce both apoptotic and anti-apoptotic signals (Solhaug et al. 2004). Diesel exhaust particles induced apoptosis in macrophages through reactive oxygen species generation, with subsequent activation of caspase cascades, loss of membrane integrity, and DNA damage (Hiura et al. 1999). Ohtoshi et al

(1998) showed that B[a] P, one of the important aromatic hydrocarbons contained in ROFA, induced release of GM-CSF and IL-8 from human airway epithelial cells.

Particles of the ROFA used in this study contain toxic elements and several PAHs with known toxic potential. These findings indicate that we dealt with a complex sample that hinders the adequate characterization of the agent responsible for the observed effects, since synergic effects are expected to occur in such cases.

Indeed, this is a common scenario in ambient particle toxicity studies, because several compounds are present in the same sample, making it virtually impossible to ascribe the responsibility for the adverse effects to a single agent.

In our study we observed substantial airway remodeling, inflammatory infiltration, and increased pulmonary responsiveness induced by OVA exposure. There was an increase in epithelial area, the area occupied by secretory cells, and the amount of both neutral and acidic mucus. There was also an increase in the density of eosinophils and macrophages in peribronchovascular infiltrate. The additional effects induced by ROFA exposure in this experimental model were small. We did not observe differences in the amount of mucus stored in the epithelium and in the density of inflammatory cells in the bronchovascular space, and we observed a small but statistically significant decrease in the area of ciliated cells. In contrast, ROFA exposure resulted in an additional increase in pulmonary responsiveness to inhaled Mch in mice exposed to OVA.

In conclusion, ROFA administration to nonsensitized mice resulted in an increase in pulmonary responsiveness and airway epithelial remodeling. Intranasal ROFA administration also amplified these responses in OVA-exposed mice but did not increase pulmonary inflammatory cell infiltration.

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