Differences in allergic inflammatory responses in murine lungs: comparison of PM2.5 and coarse PM collected during the hazy events in a Chinese city

Miao He, Takamichi Ichinose, Seiichi Yoshida, Fumiko Shiba, Keiichi Arashidani, Hirohisa Takano, Guifan Sun & Takayuki Shibamoto

To cite this article: Miao He, Takamichi Ichinose, Seiichi Yoshida, Fumiko Shiba, Keiichi Arashidani, Hirohisa Takano, Guifan Sun & Takayuki Shibamoto (2016) Differences in allergic inflammatory responses in murine lungs: comparison of PM2.5 and coarse PM collected during the hazy events in a Chinese city, Inhalation Toxicology, 28:14, 706-718, DOI: 10.1080/08958378.2016.1260185

To link to this article: https://doi.org/10.1080/08958378.2016.1260185

Published online: 05 Dec 2016.

Article views: 220

View related articles

View Crossmark data

Citing articles: 8 View citing articles
Differences in allergic inflammatory responses in murine lungs: comparison of PM2.5 and coarse PM collected during the hazy events in a Chinese city

Miao He1,2, Takamichi Ichinose2, Seiichi Yoshida2, Fumiko Shiba2, Keiichi Arashidani3, Hirohisu Takano4, Guifan Sun1, and Takayuki Shibamoto5

1School of Public Health, China Medical University, Shenyang, China, 2Department of Health Sciences, Oita University of Nursing and Health Sciences, Oita, Japan, 3Department of Immunology and Parasitology, School of Medicine, University of Occupational and Environmental Health, Fukuoka, Japan, 4Environmental Health Division, Department of Environmental Engineering, Graduate School of Engineering, Kyoto University, Kyoto, Japan, and 5Department of Environmental Toxicology, University of California, Davis, CA, USA

Abstract

Urban particulate matter (PM) is associated with an increase in asthma. PM2.5 (<2.5 μm) and coarse PM (CPM: PM2.5–PM10 μm) were collected from the air in a Chinese city during haze events. The amounts of polycyclic aromatic hydrocarbons (PAHs) were higher in PM2.5 than in CPM. Conversely, microbial elements LPS and β-glucan were much higher in CPM than in PM2.5. Concentrations of Si, Al, Fe, and Ti in CPM were greater than in PM2.5, while Pb, Cu and As concentrations were lower than in PM2.5. When RAW264.7 cells were treated with PM2.5 and CPM, the pro-inflammatory response in the cells was associated with the microbial element levels and attenuated partly by both polymyxin B (PMB) and N-acetylcystein (NAC). The expression of the oxidative stress response gene heme oxygenase1 was associated with PAHs levels. The exacerbating effects of the two-types of PM on murine lung eosinophilia were compared to clarify the role of toxic materials. When BALB/c mice were intratracheally instilled with PM2.5 or CPM (total 0.4 mg) + ovalbumin (OVA), both exacerbated lung eosinophilia along with allergy-relevant biological indicators, such as OVA-specific IgE in serum; enhancement of lung pathology when compared with counterpart samples without OVA. The exacerbating effects were greater in microbial element-rich CPM than in organic chemical-rich PM2.5. These results indicate that microbial elements have more potently exacerbating effects on the development of lung eosinophilia than do organic chemicals. In addition, oxidative stress and transition metals might be associated with the exacerbation of this negative effect.

Keywords
PM2.5, coarse PM, lung eosinophilia, microbial elements, polycyclic aromatic hydrocarbons, metals

History
Received 10 August 2016
Revised 17 October 2016
Accepted 9 November 2016

Introduction

In China, rapid industrialization, urbanization and population growth has caused a remarkable increase in air pollution, along a similar trajectory to that previously experienced by other developed countries (Seinfeld, 2004). In the first quarter of 2013, China experienced extremely severe and persistent haze pollution, affecting 1.3 million km² and 800 million people (Huang et al., 2014). One of the most harmful air pollutants is particulate matter (PM), including PM2.5 (aerodynamic diameter of less than or equal to 2.5 μm) and PM10 (aerodynamic diameter of less than or equal to 10 μm). The threat posed by this ambient particulate matter to health is one of the greatest, and still growing, public concerns (Baccarelli et al., 2014; Chen et al., 2013; Li & Liu, 2014; Lu et al., 2015; Qiao et al., 2014).

Epidemiological studies reported that the smog episodes are consistently and statistically significantly associated with higher total mortality and mortality from respiratory and cardiovascular diseases (Ping, 2015; Zhou et al., 2014). Asthma, especially in children, is the most common chronic respiratory disease worldwide, and is characterized by chronic allergic inflammation of the airways, with superimposed episodes of acute inflammation (Shadie et al., 2014). Acute exacerbations are the most clinically significant feature of asthma and are typified by increased distal airway inflammation, with exaggeration of symptoms such as cough, chest tightness and dyspnea (Dougherty & Fahy, 2009). There is substantial epidemiological evidence indicating a link between asthma morbidity including worsening asthma symptoms, deterioration in lung functions, increased emergency department visits and hospital admission, with outdoor air pollution levels (Esposito et al., 2014; Liu et al., 2014; Pan et al., 2014; Park et al., 2013).
Urban dust, which includes PM2.5 μm and coarse PM (CPM: >PM2.5–PM10 μm), encompasses within it a chemically heterogeneous mix of solid dust and anthropogenic particles emitted from fossil fuel combustion including polycyclic aromatic hydrocarbons (PAHs). The different chemical and biological components within PM2.5 and CPM may result in varying effects on specific health endpoints (Gehring et al., 2015; Strak et al., 2012). Experimental studies have shown that smaller particles (TiO2, carbon black) induce stronger pulmonary inflammatory or allergic inflammatory effects than tested at equal mass dose with their larger counterparts, due to their larger surface area to mass ratio (de Haar et al., 2006; Singh et al., 2007). Although PM surface area is an important determinant of PM toxic reactivity, the chemical composition of PM is also important (Steenhof et al., 2011). In ambient air pollution particles, CPM reportedly triggered a stronger inflammatory response than fine particles, due to endotoxin (LPS) or other contaminants adsorbed onto the particles (Jalava et al., 2015; Schins et al., 2004, Steenhof et al., 2011). On the other hand, our previous experimental research has shown that urban dust collected from the air in Beijing, China exacerbated ovalbumin (OVA)-associated murine lung eosinophilia, and that PM2.5-rich dust collected from the air in Fukuoka, Japan did as well (He et al., 2010, 2015). However, the precise exacerbating factors contained in urban dusts are not fully understood. Therefore, comparative study to clarify the role of toxic materials present in the two-types of PM in murine lung eosinophilia exacerbation is in order.

In the present study, urban PM2.5 and CPM collected during haze events of Northeast China in the winter season were used. The exacerbating effects of PM2.5 and CPM on OVA-induced allergic inflammation in murine lungs were compared to clarify the role of the chemicals and microbial materials in the two types of PM. Pathologic changes in the airway, cytological alterations, changes of inflammatory cytokines and chemokines in BALF, and OVA-specific immunoglobulin E (IgE) and IgG1 antibodies in serum were investigated in BALB/c mice. Additionally, an in vitro study investigated the gene expression of pro-inflammatory mediators and heme oxygenase1 in mouse macrophage cells (RAW264.7) exposed to PM2.5 or CPM with and without polymyxin B (LPS inhibitor) or N-acetylcystein (antioxidant). This study was performed to clarify the effects of inflammatory and oxidative stress responses by both types of PM.

Materials and methods

Animals

BALB/c mice (7 weeks of age) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Mice were checked for abnormal body weight or sickness for 1 week, and then used at 8 weeks of age. They were fed a commercial diet CE-2 (CLEA Japan, Inc., Tokyo) and given water ad libitum. Mice were housed in plastic cages lined with soft wood chips. The cages were placed in an air conditioned room at 23 °C with 55–70% humidity and a light/dark (12 h/12 h) cycle. The study adhered to the U.S. National Institutes of Health Guidelines for the use of experimental animals. The animal care method was approved by the Animal Care and Use Committee at Oita University of Nursing and Health Sciences in Oita, Japan.

Sample collections of PM2.5 and CPM

The present study used PM2.5 and CPM collected using a four-stage multi nozzle cascade impactor (MCI) (Tokyo Dylec Co.) at China Medical University on 21–25 November 2013, when a massive haze event was occurring in Shenyang, China.

The atmospheric levels of the PM2.5 and PM10 on 21–25 November were 114–225 μg/m³ and 167–287 μg/m³, respectively. The levels of size-classified mass and the elemental concentrations of PM2.5 were measured by an MCI combined with the 1st, 2nd and 3rd stages with 12-orifice and back-up stage according to a previously reported method (Ma et al., 2012). PM was sampled directly on substrate materials placed behind the jet-nozzles of a MCI with the airflow at 20 L/min. The size cutoffs for PM at each stage were: 1st stage – giant fraction (>PM10), 2nd stage – coarse fraction (PM2.5–10), 3rd stage – fine fraction (PM0.5–2.5), and a back-up stage fraction (<PM0.5). The filter used was a non-hole Nuclepore® polycarbonate filter (47 mm diameter) for the 1st, 2nd, and 3rd stages and a 0.01 μm hole filter for the back-up stage. Particles were removed from the coarse fraction and the fine fraction using a sterilized stainless steel spatula. The particle weights collected from each Teflon filter was measured. They were then pooled into one sterilized dry bottle. The particle samples collected from each fraction were stored at −30 °C in a germ-free case with a desiccant until use for the experiments and analysis.

Analysis of particle size

The particle sizes of PM were analyzed with a KEYENCE all in one BZ-9000 fluorescence microscope (Osaka, Japan). A total of 5798 particles were counted. PM2.5 ranged from 0.5 to 4 μm, and CPM ranged from 0.5 to 10 μm. The median diameter was 0.87 ± 0.59 μm (M ± SD) for PM2.5 and 1.48 ± 1.65 μm (M ± SD) for CPM.

Analysis of components in the samples

The elements in the samples were analyzed by inductively-coupled plasma atomic emission spectroscopy (ICP-AES, 61E Trace and ICP-750, Thermo Jarrell-Ash, MA) after acid digestion with mixed acids (68% nitric, 38% hydrofluoric, and 70% perchloric = 5:1:1) was performed at 180 °C for 3 h. The water soluble components in the samples were analyzed using an ion chromatograph (DX-100, Dionex, Sunnyvale, CA) and ICP-AES (61E Trace, Thermo Jarrell-Ash).

Analysis of microbial elements in particles

Each particle sample was measured by kinetic assay using Endospec ES test MK (Seikagaku Corp., Tokyo, Japan) for LPS activity and by Fungitec G test MK (Seikagaku Corp., Tokyo, Japan) for β-glucan activity. In brief, approximately 5 mg of PM2.5 or CPM was suspended in 1 ml water, which is used for LPS and β-glucan assays with the test kit (Seikagaku Corp., Tokyo, Japan) for 1 h and placed on the bench top at room temperature for 2 h. Supernatants then were recovered.
and tested for LPS and β-glucan concentrations using Pyro Color-MP: Chromogenic Diazo-Coupling Kit (Associates of Cape Cod, Inc., MA, USA) for endpoint-colorimetry. The detection limits for LPS and β-glucan were 0.001 EU/ml and 2 pg/ml, respectively.

Analysis of polycyclic aromatic hydrocarbons (PAHs) in particles

PAHs were analyzed according to a previously reported method (He et al., 2010, 2015, 2016a, 2016b; Ichinose et al., 2008). Briefly, samples collected in the Teflon filter were extracted twice with a 20 ml portion of dichloromethane at 15°C by ultrasonic extraction. The extract was filtered with an No 5C filter paper and the filtrate was allow to stand in the dark until the solvent was evaporated to dryness to yield solid materials. The residual materials were dissolved in 0.5 ml acetonitrile and analyzed for PAHs by a Hitachi Model 600 HPLC (Hitachi, Japan) equipped with a Model L-7485 fluorescence detector (Hitachi, Japan) and a 4.0 mmø × 250 mm column packed with Wakosil-II 5C 18HG (Waka Pure Chemicals Industry, Ltd., Osaka, Japan). The mobile phase was acetonitrile/water (80/20, v/v) at 1.5 ml/min. Identification of PAHs in the sample was conducted by comparison of the HPLC retention time and fluorescence/excitation spectra to those of authentic PAHs according to a previously reported method (Kodama & Arashidani, 1983). Standard PAHs were obtained from Supelco (Bellefonte, PA), Aldrich Chemical Co., Inc. (Milwaukee, WI) and Tridom Chemical Inc. (Hauppauge, NY).

In vitro study

RAW264.7 cells were cultured at 37°C in a humidified atmosphere of 5% CO2–95% air and maintained in Dulbecco’s modified Eagle’s medium with 10% heat inactivated fetal bovine serum. For gene expression analysis, cells were plated at a concentration of 4 × 10^4 cells per 60-mm dish, and then these cells were exposed to PM2.5 or CPM at doses of 30 μg/ml. In addition, PM2.5 (3 mg/ml) and CPM (3 mg/ml) were pretreated with polymixin B sulfate (PMB, Sigma-Aldrich Co., St. Louis, MO) for 1 h at 37°C. Cells were exposed to PMB-treated PM2.5 or PMB-treated CPM at the doses of 30 μg/ml. The final dose of PM2.5 was 2 μg/ml. Other cells were pre-incubated with 10 mM N-acetylcystein (NAC) for 1 h and then exposed to PM2.5 or CPM at the doses of 30 μg/ml. After 3 h, the expression levels of pro-inflammatory cytokine genes, inducible nitric oxide synthase (iNOS) and heme oxygenase 1 (Hmox1) gene in these cells were measured.

Gene expression analysis in PM-stimulated RAW264.7 cells

Total RNA was extracted by standard procedures using 0.5 ml of Isogen (Nippon Gene, Tokyo, Japan) per dish. After DNase treatment of the total RNA, cDNA was synthesized by reverse transcription using M-MLV. Quantitative PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA) under the same conditions as in our previous studies (He et al., 2010, 2012, 2016a). Two wells were used for each sample. The relative expression of each sample was calculated as the mean value divided by the mean value for Actin. The primers and probes used in this in vitro study are shown in Supplementary Table 1.

Study protocol

A total of 96 male BALB/c mice were divided into six groups (n = 16 per group) according to the following treatments with particles for investigation: sterile saline (control), PM2.5, CPM, OVA, OVA + PM2.5, and OVA + CPM. PM particles were suspended in an LPS, β-glucan-free sterile saline solution containing 0.9% NaCl for injection (Otsuka Co., Kyoto, Japan). This suspension was sonicated for 5 min with an ultrasonic disruptor, UD-201 type with micro tip (Tomy, Tokyo, Japan), under cooling conditions to make the particles disperse. OVA (Grade VII), which was purchased from Sigma-Aldrich (St. Louis, MO), was dissolved in the same sterile saline. The IT instillation dose of PM was 0.1 mg per mouse and the treatment dose of OVA was 4 μg per mouse (He et al., 2016b; Ren et al., 2014a, b). The OVA-treatment dose, administration times and periods can elucidate the potential of particles (0.1 mg) to exacerbate allergic reactions in mice. The instillation volume of the suspension was 80 μl/mouse. Mice were instilled with a mixed or individual solution of OVA, PM2.5, and CPM through a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical, Osaka, Japan) 4 times at 2-week intervals. Therefore, the total instillation dose of PM was 0.4 mg/mouse. The control group was treated with an 80 μl sterile saline solution. The one-time instillation dose of PM was set to 0.1 mg/mouse in accordance with a previously reported method (He et al., 2010, 2015, 2016a; Ichinose et al., 2008; Ren et al., 2014a, b).

Regarding the instillation dose of PM, for mice exposed to 1 mg/m3/day for 6 weeks, the resultant lung deposition amount was 454 μg (approximately 10.8 μg/day). This amount was calculated from tidal air volume (0.15 mL) and breathing rate (200 breaths/min) and a 50% deposition rate in the case of PM2.5 (James et al., 1994). The total instillation dose (0.4 mg) of particles in this study was approximately 0.88 times the amount of the lung deposition amount (454 μg).

One day after the last intratracheal administration, mice from all groups (age = 14 weeks) were euthanized by exsanguination (collection of blood from heart) under deep anesthesia by intraperitoneal injection of pentobarbital.

Pathological evaluation

Eight out of the 16 mice from each group were used for pathologic examination. Lungs were fixed in a 10% neutral phosphate-buffered formalin solution. After separation of the lobes, slices 2 mm in width were made from each lobe and embedded in paraffin, sectioned at a 3 μm thickness, and then stained with May-Grunwald’s stain solution (Nacalai tesque, Inc, Kyoto, Japan) and Giemsa’s azur eosine methylene blue solution (Merck KGaA, Darmstadt, Germany) to evaluate the degree of infiltration of eosinophils or lymphocytes in the airway from proximal to distal. Sections were also stained with periodic acid-Schiff (PAS) (Waka Pure Chemicals...
Industry, Ltd., Osaka, Japan) to evaluate the degree of proliferation of goblet cells in the bronchial epithelium. Pathological analysis of the inflammatory cells and epithelial cells in the airway of each lung lobe on the slides was performed using a Nikon ECLIPSE light microscope (Nikon Co, Tokyo, Japan).

The degree of proliferation of goblet cells in the bronchial epithelium was graded according to the following scale: 0, not present; 1, slight; 2, mild; 3, moderate; 4, moderate to marked; and 5, marked. “Slight” was defined as less than 20% of the airway infiltrated with goblet cells stained with PAS; “mild” as 21–40%; “moderate” as 41–60%; “moderate to marked” as 61–80%; and “marked” as more than 81% (He et al., 2010, 2015, 2016a; Ichinose et al., 2008). In brief, tracheas were cannulated after the collection of blood. The lungs were lavaged with two syringe injections of 0.8 ml of sterile saline

Quantitation of cytokines and chemokines in BALF

The cytokine protein levels in the BALF were determined using enzyme-linked immunosorbet assays (ELISA). Interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-13, IL-17, interferon (IFN)-γ, eotaxin, keratinocyte chemoattractant (KC), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, regulated upon activation normal T-cell expressed, and presumably secreted (RANTES), tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β and cyclooxygenase (Cox)-2 were measured using ELISA kits from R&D Systems Inc. (Minneapolis, MN). IL-5 and IL-12 were measured by ELISA kits from Thermo Scientific (Rockford, IL). MCP-3 was measured with an ELISA kit from Bender Med Systems (Burlingame, CA).

BALF samples (50 μl/well) were added to a plate with 96 wells. The ELISA for IL-1β, IL-4, IL-6, IL-13, IL-17A, IL-22, IL-33, eotaxin, KC, MCP-1, MIP-1α, RANTES, TNF-α, IFN-γ IL-5, IL-12 and MCP-3 was conducted using matching antibody pairs according to the manufacturer’s instructions. The second antibodies were conjugated with horseradish peroxidase. Subtractive readings of 550 nm from the reading at 450 nm were converted to picograms/ml (pg/ml) using the values obtained from standard curves prepared with varying concentrations of recombinant IL-1β (<2.31 pg/ml), IL-4 (2 pg/ml), IL-6 (1.6 pg/ml), IL-13 (1.5 pg/ml), IL-17A (5 pg/ml), IL-22 (8.2 pg/ml), IL-33 (6.85 pg/ml), eotaxin (3 pg/ml), KC (2 pg/ml), MCP-1 (2 pg/ml), MIP-1α (1.5 pg/ml), RANTES (2 pg/ml), TNF-α (1.88 pg/ml), IFN-γ (2 pg/ml), IL-5 (5 pg/ml), IL-12 (12 pg/ml) and MCP-3 (2.6 pg/ml). The values in parentheses were the limits of detection.

Antigen-specific IgE and IgG1 antibodies

OVA-specific IgE and IgG1 antibodies were measured using a Mouse OVA-IgE ELISA kit and a Mouse OVA-IgG1 ELISA kit (Shibayagi Co, Shibukawa, Japan). According to the manufacturer’s protocol, 1 U of anti-OVA IgE is defined as 1.3 ng of antibody, and 1 U of anti-OVA IgG1 as 160 ng of antibody. The absorption at 450 nm (sub-wave length, 620 nm) for OVA-specific IgE and IgG1 antibodies was measured using a microplate reader (Spectrafluor, Tecan, Salzburg, Austria).

Statistical analysis

Statistical analyses of gene expression in RAW264.7 cells were conducted by the one-way analysis of variance (ANOVA)-test. Statistical analyses of cell numbers (macrophages, neutrophils, eosinophils and lymphocytes) and cytokine/chemokine proteins in BALF, OVA-specific IgE and IgG1 antibodies in serum as well as pathological score were conducted by the Tukey Test for Pairwise Comparisons, after the t-test and ANOVA-test were performed. All the analyses were performed with KyPlot Ver.5, Kyens Lab Inc., Tokyo, Japan. Differences among groups were determined as statistically significant at a level of p < 0.05.

Results

Components found in PM samples

In CPM, the concentration of Si (105 μg/mg) was the highest, followed by Ca (53 μg/mg), Fe (35 μg/mg), Al (31 μg/mg), and Ti (2.5 μg/mg). Concentrations of these major elements were generally greater in CPM than in PM2.5. OC1 and OC2 were found at much higher levels in PM2.5 (9.8 and 29 μg/mg, respectively) than in CPM (0.84 and 11 μg/mg, respectively). Also EC1 was at higher level in PM2.5 (160 μg/mg) than in CPM (53 μg/mg) (Supplementary Table 2).

In CPM, Mn had the highest concentration (900 ng/mg), followed by Ba (600 ng/mg), Cu (260 ng/mg), Cr (230 ng/mg) and As (170 ng/mg). In PM2.5, Mn had the highest concentration (760 ng/mg), followed by As (500 ng/mg), Cu (440 ng/mg), Cr (150 ng/mg), Ba and Mo (140 ng/mg). In contrast to the major elements, the differences in the presence of the minor elements in the two samples were varied (Supplementary Table 3).
The concentration of \( \text{SO}_4^{2-}, \text{Cl}^-, \text{NH}_4^+, \text{Na}^+, \text{Mg}^{2+}, \text{and Ca}^{2+} \) was higher in PM2.5 than in CPM. On the other hand, \( \text{NO}_3^-, \text{K}^+, \text{Mg}^{2+}, \text{and Ca}^{2+} \) were higher in CPM than in PM2.5. The levels of LPS and \( \beta \)-glucan were also higher in CPM (330 pg/mg, 880 pg/mg, respectively) than in PM2.5 (10 pg/mg, 210 pg/mg, respectively). CPM had a 33-fold higher level of LPS than that in PM2.5, and a 4.23-fold higher level of \( \beta \)-glucan (Supplementary Table 4).

A total of 14 PAHs was identified. PAHs were found at much higher levels in PM2.5, ranging from 588 \( \mu \text{g/g} \) (dibenzo[a,h]anthracene) to 17.8 \( \mu \text{g/g} \) (coronene), than in CPM, where they ranged from 223 \( \mu \text{g/g} \) (dibenzo[a,h]anthracene) to 6.0 \( \mu \text{g/g} \) (coronene) (Supplementary Table 5).

**Differences of gene expression in RAW264.7 treated with two types of PM**

To clarify the effect of the inflammatory and oxidative stress responses of the two types of PM, pro-inflammatory cytokine genes, iNOS gene and \( \text{Hmox}1 \) gene in RAW264.7 cells were measured with and without PMB or NAC (Figure 1). PM2.5-stimulated RAW264.7 cells significantly increased the expression of \( \text{IL}-1\beta \), \( \text{TNF}-\alpha \), \( \text{MIP}-1\alpha \), iNOS, and \( \text{Hmox}1 \) genes compared with those of control. CPM-stimulated the cells significantly increased all genes (\( \text{IL}-1\beta \), \( \text{MCP}-1 \), \( \text{TNF}-\alpha \), \( \text{MIP}-1\alpha \), iNOS) expression compared with the control. The gene expression levels in CPM were much higher than those in PM2.5.

On the other hand, PM2.5-induced IL-1\( \beta \) gene expression was inhibited 64% by PMB and 28% by NAC, and the CPM-induced gene was inhibited by 57% and 17%, respectively. The PM2.5-induced MCP-1 gene expression was prevented completely by PMB and NAC, but CPM-induced gene expression was prevented 36% by PMB and 52% by NAC. PM2.5-induced TNF-\( \alpha \) gene expression was prevented 38% by PMB and 31.5% NAC, and CPM-induced gene expression was prevented 30% and 28%, respectively. PM2.5-induced expression of MIP-1\( \alpha \) gene was prevented 38% by PMB and 32% by NAC, and CPM-induced gene expression was prevented about 20% by PMB and CPM. PM2.5- and CPM-induced iNOS gene expression was prevented 29% and 37% by PMB, respectively, but the gene expression was increased by NAC. Thus, these inflammatory responses were reduced at least partly by PMB and NAC. On the other hand, the \( \text{Hmox}1 \) gene expression, a marker of oxidative stress, was higher in PM2.5 than in CPM. The expression was markedly reduced by NAC, but the gene expression in CPM was increased by PMB.

**PM2.5 and CPM enhanced pathologic changes in the airway**

To confirm the effects of PM on airway inflammation and goblet cell proliferation caused by OVA, lung pathology in the six groups of mice was examined. Figure 2 shows an airway stained by PAS. Figure 3 shows airway submucosa stained by...
May-giemsa. No pathologic alterations were found in the lungs of the control group (Figures 2A and 3A). PM2.5 and CPM alone caused very slight proliferation of goblet cells in the bronchial epithelium (Figure 2B and C) and slight infiltration of neutrophils and lymphocytes into the airway submucosa (Figure 3B and C). Treatment with OVA alone caused slight goblet cell proliferation (Figure 2D) in the bronchial epithelium and very slight infiltration of eosinophils into the airway submucosa (Figure 3D). OVA + PM2.5 caused moderate goblet cell proliferation in the airways (Figure 2E). OVA + CPM caused more prominent goblet cell proliferation (Figure 2F). OVA + PM2.5 caused moderate infiltration of neutrophils and eosinophils into connective tissues in the airway (Figure 3E). OVA + PM2.5 also caused marked

Figure 2. Pathological changes in the airway epithelium of mice treated with PM2.5 or CPM. (A) Control, (B) PM2.5, (C) CPM, (D) OVA, (E) OVA + PM2.5, (F) OVA + CPM. A–F: PAS stain. Arrows show goblet cells with mucin in the airway epithelium. Thin arrows show infiltration of inflammatory cells.

Figure 3. Infiltration of inflammatory cells in the airway of mice treated with PM2.5 or CPM. (A) Control, (B) PM2.5, (C) CPM, (D) OVA, (E) OVA + PM2.5, (F) OVA + CPM. (A–F) May-giemsa stain. Arrows show eosinophils with red granules. Thin arrows show neutrophils. Triangles show macrophages.
infiltration of eosinophils into the airway submucosa (Figure 3F).

Figure 4 shows the lung pathology scores. Both co-treatments significantly increased goblet cell proliferation in the airway compared with their single (PM2.5- or CPM-only) counterparts. OVA + PM2.5 and OVA + CPM also significantly increased eosinophil infiltration in the airway compared with their single counterparts. These pathological changes were greater in the OVA + CPM group than in the OVA + PM2.5 group. Both co-treatments also caused moderate to marked accumulation of lymphocytes in the airways. The degree of accumulation was greater in the OVA + CPM group than in the OVA + PM2.5 group.

**PM2.5 and CPM increased BALF cell numbers**

To evaluate the effects of the PM on lung inflammation induced by OVA, the cellular profile of BALF was investigated (Figure 5). PM2.5 alone significantly increased macrophage numbers more than the control and CPM alone significantly increased total cell and neutrophil numbers compared with the controls. Furthermore, combined administration of OVA + PM2.5 and OVA + CPM significantly increased total cell, eosinophil and lymphocyte numbers compared with the controls, OVA, PM2.5 or CPM alone. OVA + CPM resulted in a remarkable elevation of neutrophils compared with the controls, OVA and OVA + PM2.5. In addition, OVA + PM2.5 significantly increased macrophages compared with the controls, OVA and PM2.5 groups. OVA + CPM significantly increased the number of macrophages compared with the controls and the OVA group. The inflammatory cell numbers in the groups with and without OVA were higher in CPM than in PM2.5. However, there were no significant differences between PM2.5 and CPM except for neutrophils in OVA + PM2.5 versus OVA + CPM.

**PM2.5 and CPM-enhanced cytokine and chemokine levels in BALF**

To investigate the effects of PM on lung inflammation and OVA-induced lung eosinophilia, the protein levels of cytokines and chemokines in BALF were measured (Figures 6–8).

PM2.5 alone significantly increased IL-12 compared with the control, and CPM alone increased IL-12, TNF-α and KC compared with the controls. The increased levels of IL-12, TNF-α and KC were higher in CPM than in PM2.5.

OVA + PM2.5 markedly increased IL-12 and MIP-1α compared with the controls and OVA. Also OVA + PM2.5 significantly elevated Th-2 cytokines IL-4 compared with the control, OVA, PM2.5; IL-5, IL-6, IL-13, and IL-10 compared with the controls, OVA and PM2.5 alone. The increased levels of IL-4, IL-5, IL-6, IL-10 and IL-13 were higher in PM2.5 than in CPM.
OVA + CPM strikingly augmented expression of IL-1β, IL-5, IL-13, IL-12, TNF-α, eotaxin and MIP-1α compared with the controls and OVA. OVA + CPM remarkable induced expression of MCP-1 compared with the control, OVA, PM2.5; MCP-3 compared with the control, OVA, PM2.5 and IL-10 compared with the control, CPM alone, and OVA. The increased levels of IL-1β, KC, MCP-1, MCP-3 and eotaxin were higher in CPM than in PM2.5.

IL-17, INF-γ and TGF-β were not detected in any groups tested (data not shown).

PM2.5 and CPM enhanced serum OVA-specific IgE and IgG1

To examine whether PM have adjuvant activity on antigen specific Ig production, OVA-specific IgE and IgG1 antibodies were measured (Figure 9). Co-exposure with OVA and PM2.5/CPM significantly increased OVA-specific IgE and IgG1 production compared with the controls, and the OVA-only group. Furthermore, OVA + CPM significantly elevated OVA-specific IgG1 production compared with OVA + PM2.5.

Discussion

The present in vitro study showed that the pro-inflammatory response to the two-types of PM investigated was associated with the microbial element levels (LPS, β-glucan). Both PM effects were attenuated remarkably by PMB or NAC, suggesting that LPS present in PM and oxidative stress may contribute greatly to inflammatory gene expressions. The expression of oxidative stress response gene Hmox1 was associated with PAHs levels in PM. The high level of oxidative stress caused by PAHs activates the NF-κB and MAPKs signaling cascades, which are important for the expression of many genes that participate in pulmonary inflammation (Li et al., 2003). Therefore, oxidative stress generated from PAHs may contribute at least partly to the expression of inflammatory gene in vitro.

In the current in vivo study, the single exposure to an equal dose of PM2.5 or CPM caused lung pathologies such as bronchitis and alveolitis as assessed by histological observation and an increase of neutrophils and their relevant pro-inflammatory mediators KC, IL-12, and TNF-α in BALF. It is well known that TNF-α contributes to host defense against bacterial invasion, activates macrophages to kill intracellular pathogens, and is directly involved in neutrophilic inflammation of the airways (Kips et al., 1992; Willingham et al., 2007; Windsor et al., 1993). KC (IL-8 in humans) recruits and activates neutrophils (Matsushima, 1994). These increases were much higher in CPM than in PM2.5. Other pro-inflammatory mediators (IL-6, MCP-1, MIP-1α, RANTES) in CPM also were tended to increase more than in PM2.5. Similar observations in vitro (Jalava et al., 2007, 2008) and
in vivo (Happo et al., 2010; Schins et al., 2004), that inflammatory responses were stronger in large particles than in small particles in ambient PM, have been reported. This inflammatory response may be caused at least partly by LPS present in PM. Although oxidative stress generated from PAHs in PM2.5 might be related to induction of lung inflammation in vivo, the magnitude of inflammatory response may depend on the quantities of microbial elements present in PM rather than on their chemical composition or particle size.

The combined exposure to OVA and an equal dose of PM2.5/CPM caused exacerbation of lung eosinophilia, including enhancement of eosinophil recruitment in the submucosa of the airway, goblet cell proliferation in the bronchial epithelium, remarkable increases of eosinophils and Th2 cytokines (IL-4, IL-5, IL-13) and eosinophil-relevant chemokines (MCP-3, Eotaxin) in BALF, and an increase of serum OVA-specific IgG1 and IgE compared with the PM2.5/CPM-only counterparts. These Th2-derived cytokines and chemokines are key mediators in triggering the symptoms of asthma and are critical for the recruitment and survival of eosinophils (Foster et al., 1996), the production of antigen specific antibodies (Mosmann & Coffman, 1989), and the production of mucous cells, such as goblet cells, in the bronchial epithelium (Tesfaigzi, 2008). The exacerbating effect on lung eosinophilia is greater in the microbial element-rich CPM than in the organic chemical-rich PM2.5. However, an increase of Th2 cytokines IL-4, IL-5, and IL-13 in BALF was higher in PM2.5 than in CPM, contrary to an increase of eosinophils in BALF and lung pathology. This phenomenon may be indicative of a time lag. The inductions of Th2 cytokines might be occurring at an earlier phase.

Regarding exacerbating factors on allergic lung inflammation, we previously reported that co-exposure to heated ASD (H-ASD) as mineral dust – heated at 360°C to exclude toxic materials, such as microbial materials and PAHs, prior to application – and trace ultrapure LPS from Escherichia coli enhances Th2 responses to OVA in a mouse asthma model (Ren et al., 2014b). Exposure to LPS is a significant risk factor for increased asthma prevalence and severity (Alexis et al., 2004). Previous in vivo findings have indicated that low levels of LPS could cause Toll like receptor (TLR) 4-dependent Th2 responses to OVA (Dabbagh et al., 2002; Eisenbarth et al., 2002) through MyD88 (Piggott et al., 2005). TLRs are principal innate immune sensors recognizing microbial pathogen-associated molecular patterns and endogenous danger molecules (Krejsek et al., 2005) released from host cells. TLR2 is a receptor for β-glucan of fungi or peptidoglycan of Gram-positive bacteria (Beutler, 2004) and TLR4 is a receptor for LPS (Schwandner et al., 1999). We also reported that co-exposure to Bjercandera adusta (hyphae and spore) containing TLR2-ligand β-glucan, which is inactivated with 1% formalin, H-ASD and OVA aggravated lung eosinophilia compared with H-ASD + OVA (Liu et al., 2014). TLR2-ligand like Pam3Cys can activate OVA-associated Th2-biased immune response in experimental asthma (Redecke et al., 2004). More recently, we have reported that crude ASD induced TLR2 and TLR4 signal to

Figure 6. Expression of IL-1β, IL-6, IL-10, IL-12 and TNF-α in bronchoalveolar lavage fluid (BALF). All values are expressed as mean ± SE (n = 8). *p < 0.05 versus Control, †p < 0.05 versus PM2.5, ‡p < 0.05 versus CPM, §p < 0.05 versus OVA.
trigger Th2-dominant lung allergic inflammation via a MyD88-dependent signaling pathway. The TLR4 ligand-LPS and TLR2 ligand like β-glucan in crude ASD may be strong candidates for exacerbation of lung eosinophilia (He et al., 2016a). In another recent study, urban PM2.5 also was seen to have caused similar TLR2 and TLR4 signals in a mouse model of asthma (unpublished data). In the current study, therefore, it is likely that the microbial elements LPS or β-glucan present in PM may play an important role in the exacerbation of lung eosinophilia. The differences in quantities of microbial elements present in PM may be due to differences of allergic inflammatory responses in particles. However, there is a report that only histopathological lesions in the lungs of mice intranasally administrated coarse or fine mixed (3 mg/ml) PM with OVA (0.4 mg/ml) exhibited a significant difference in effects between coarse and fine (coarse > fine) PM; no associations were found between the endotoxin content and the biological effects parameters, although endotoxin was much more confined to the coarse fraction (Steerenberg et al., 2004). Although the reasons for the differences between this report and our evidences are not fully clear at this point, it may be due to differences in the particle instillation dose. It is well known that high doses of LPS cause Th1-dominant inflammation and suppress allergen induced Th2 response (Eisenbarth et al., 2002).

On the other hand, we previously reported that co-exposure to Tar, which is soluble organic fraction (containing organic carbon such as PAHs derived from fossil fuel combustion) extracted from air dust sample with dichloromethane, H-ASD and OVA exacerbated murine lung eosinophilia compared to H-ASD + OVA or Tar + OVA. Therefore, we concluded that particles coexisting with organic chemical could exacerbate allergic inflammatory responses by producing antigens (Ren et al., 2014a). In the present study, however, the exacerbating effect of lung eosinophilia by PM2.5 was smaller than in CPM, suggesting that organic chemical-rich fine particles may have only a weak on their exacerbation compared with microbial element-rich mineral particles. We recently have reported a similar observation in a comparative study between chemical-rich urban PM2.5 and β-glucan-rich desert fine particle (He et al., 2016b).

Na, Si, Al, K, Ca, Fe, Ti, Sc, Mn, and Ba are generally known as seawater and soil components (Mori et al., 2002).
In this study, these elements were higher in CPM than in PM2.5. Zn, Pb, As, organic carbon (OC), elemental carbon (EC), SO$_4^{2-}$/CO$_3^-$, NO$_3^-$ and NH$_4^+$ are produced by anthropogenic pollution, such as fossil fuel combustion, and are derived from industrial and vehicular emissions (Primbs et al., 2007).

These constituents were higher in PM2.5 than in CPM, except for NH$_4^+$ in the test samples. However, it is unclear whether the metals and OC in the particle are actually related to either exacerbation of lung inflammation or allergic inflammation in the present *in vivo* study.

In this study, these elements were higher in CPM than in PM2.5. Zn, Pb, As, organic carbon (OC), elemental carbon (EC), SO$_4^{2-}$/CO$_3^-$, NO$_3^-$ and NH$_4^+$ are produced by anthropogenic pollution, such as fossil fuel combustion, and are derived from industrial and vehicular emissions (Primbs et al., 2007).

These constituents were higher in PM2.5 than in CPM, except for NH$_4^+$ in the test samples. However, it is unclear whether the metals and OC in the particle are actually related to either exacerbation of lung inflammation or allergic inflammation in the present *in vivo* study.

---

Figure 8. Expression of IL-4, IL-5, IL-13, eotaxin and MCP-3 in bronchoalveolar lavage fluid (BALF). All values are expressed as mean ± SE (n = 8). *p < 0.05 versus Control, †p < 0.05 versus PM2.5, ‡p < 0.05 versus CPM, ‡‡p < 0.05 versus OVA.

Figure 9. Effect of PM2.5 and CPM on OVA-specific IgE and IgG1 production in serum. All values are expressed as mean ± SE (n = 8). *p < 0.05 versus Control, †p < 0.05 versus PM2.5, ‡p < 0.05 versus CPM, ‡‡p < 0.05 versus OVA, ‡§p < 0.05 versus OVA + PM2.5.
Regarding other constituents in PM, our previous work has shown negative associations between extra SO$_4^{2-}$ in soil dust and the exacerbation of mite allergen-induced lung eosinophilia in mice (Hiyoshi et al., 2005). Also TiO$_2$ exhibited negative effects (Ichinose et al., 2008). The main element of soil dust, amorphous silica (SiO$_2$), exhibited positive effects (Ichinose et al., 2008), but H-ASD containing 60–80% crystalline silica appeared to have only a weak effect on OVA-induced lung eosinophilia exacerbation (Liu et al., 2014; Ren et al., 2014b).

**Conclusion**

Urban PM2.5 and CPM collected during haze events in a Chinese city exacerbated allergen-induced lung eosinophilia. The exacerbating effect is greater in microbial element-rich CPM than in organic chemical-rich PM2.5. Microbial element contamination of PM may play an important role in such exacerbation. Therefore, exposure to CPM as well as PM2.5 is a significant risk factor for inflammatory and allergic lung diseases. Further investigation into whether urban PM containing transition metals (i.e. Fe, Cu, Ni) and/or oxidative stress cause exacerbation of murine lung eosinophilia using metals chelate chemicals, or NAC of antioxidants is in order. Our findings may serve as a warning as to the ill effects of urban dust on the human respiratory system.

**Acknowledgements**

This study was supported by grants from the Global Environment Research Fund (5-1457) of the Ministry of the Environment Japan and National Nature Science Foundation of China (81302403). We appreciate the vital contribution of students at Oita University of Nursing and Health Sciences in this research.

**Declaration of interest**

The authors declare that they have no competing interests.

**References**


Park M, Luo S, Kwon J, et al. (2013). Effects of air pollution on asthma hospitalization rates in different age groups in metropolitan cities of Korea. Air Qual Atmos Health 6(3).


Steenshoof M, Gosens I, Strak M, et al. (2011). In vitro toxicity of particulate matter (PM) collected at different sites in the Netherlands is associated with PM composition, size fraction and oxidative potential—the RAPTES project. Part Fibre Toxicol 8:26.


Supplementary material available online