

RESEARCH ARTICLE

Urban particulate matter in Beijing, China, enhances allergen-induced murine lung eosinophilia

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Abstract

It has been reported that ambient particulate matter (PM) in some large cities, such as Beijing, China, causes adverse respiratory health effects. However, there is currently no experimental report on the relationship between bronchial asthma and urban PM (UPM) in northeast Asia. In this study, the microbial and chemical substances adsorbed onto UPM collected in Beijing were excluded by heat-treatment at 360°C for 30 min. The effects of UPM or heated UPM (H-UPM) toward allergic lung inflammation were compared in murine lungs to investigate the role of organic substances. ICR mice were administered intratracheally with the two kinds of UPM and/or ovalbumin (OVA) 4 times at 2-week intervals. UPM and H-UPM enhanced eosinophil recruitment induced by OVA in the alveoli and in the submucosa of the airway, which has a goblet cell proliferation in the bronchial epithelium. UPM and H-UPM synergistically increased Th-2 cytokines—interleukin (IL)-4 and IL-13, eosinophil-relevant cytokines and chemokines, such as IL-5 and monocyte chemotactic protein-3 (MCP-3), induced by OVA in bronchoalveolar lavage fluid (BALF). The enhancing effects were much greater in UPM than in H-UPM. UPM induced adjuvant effects on specific immunoglobulin E (IgE) and IgG1 production by OVA. In an in vitro study using RAW264.7 cells, UPM increased the expression of Toll-like receptor 2 (TLR2) mRNA, but not TLR4 mRNA. H-UPM caused no expression of both TLR mRNAs. These results suggest that the aggravated lung eosinophilia in UPM was due to activation of a Th2-associated immune response via the activation of TLR2 by microbial materials. Chemical materials of air pollutant origin contained in UPM, and inorganic components (elemental carbon, mineral elements) in H-UPM, could also cause the aggravation.

Keywords: Allergen; Beijing; lung eosinophilia; murine; particulate matter

Introduction

Previous epidemiologic studies have indicated that exposure to ambient particulate matter (PM) in a metropolitan area closely relates to increases in mortality and morbidity of cardiovascular and respiratory diseases (Dominici et al., 2006; Kan et al., 2007; Pope, 2004; Samet et al., 2000; Stieb et al., 2002). PM_{2.5} (particle sizes less than 2.5 µm) and PM₁₀ (particle sizes of 2.5–10 µm) have been associated with an increase of asthma patients (Schwartz et al., 1993), including childhood asthma (Romieu et al., 1996). Experimental studies have reported that exposure to ambient PM near a heavily trafficked Los Angeles freeway

enhanced inflammatory and allergic responses in ovalbumin (OVA)-sensitized BALB/c mice (Kleinman et al., 2007).

PM_{2.5} and PM₁₀ contain a variety of particles, such as industrial emission, vehicular emission (e.g., diesel soot), coal combustion, sea salt, and soil dust (Houghton et al., 2001). Ambient air pollution in large cities in China has changed from the conventional coal combustion type to the mixed coal combustion/motor vehicle emission type (Chen et al., 2004). However, the chemical composition of urban PM (UPM) collected in Beijing is different from those in the USA and Europe. For example, the concentration ratios of lead and

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iron to aluminum in Beijing UPM were less than those of USA UPM. In contrast, the sodium ratio of Beijing UPM was 2–4 times higher than that of USA UPM (Mori et al., 2008). Total suspended particle (TSP) levels in large cities in northeast China sometimes can reach several hundred to almost one thousand microgram per cubic meter. TSP in Beijing is partly due to sand storms coming from middle and northwestern China (the Gobi Desert and the Ocher Plateau) (Chen et al., 2004). Asian dust aerosol spreads over large areas, including East China, the Korean Peninsula, and Japan during the spring season (Kim et al., 2001; Husar et al., 2001). Asian dust and the air-mass contain carbon, sulfate, and nitrate produced by anthropogenic pollution, such as fossil fuel combustion, derived from the industrial and vehicular emission from urban areas in Eastern China (Kim et al., 2008; Primbs et al., 2007). Therefore, a major public concern in the countries of Eastern Asia is the potentially hazardous effect on respiratory diseases caused by Asian dust aerosol containing toxic pollutants.

Recent epidemiologic studies have shown that Asian dust storm events coincide with an increase in daily admissions and clinic visits for asthma (Yang et al., 2005), allergic rhinitis in Taipei, Taiwan (Chang et al., 2006), and acute respiratory symptoms in children with mild asthma (Yoo et al., 2008). In results compatible to those of these epidemiological investigations, we have experimentally demonstrated the aggravating effects of Asian sand dust on OVA-induced experimental murine lung eosinophilia and the nasal allergic reaction induced by Japanese cedar pollen administration in guinea pigs (Hiyoshi et al., 2005; Ichinose et al., 2006, 2008a, 2008b, 2009). However, the allergic asthma effects of PM with a large amount of constituents derived from air pollutants in Northeast Asia are not clear.

In the present study, we used urban PM_{10} (UPM_{10}) collected from the atmosphere in Beijing. The UPM_{10} is the most typical urban aerosol in northeast Asia. Comparison of allergic inflammation between aggravating effects of UPM that had been excluded of toxic materials (microbial materials, sulfate, etc.) by heating, and crude UPM were performed in the murine lungs. On the other hand, Toll-like receptors (TLRs) in antigen-presenting cells are known to lead to the induction of adaptive immune responses through the recognition of microbial components (Beutler et al., 2002; Schwandner et al., 1999). In the present *in vitro* study, the expression of TLR2 and TLR4 mRNAs in RAW264.7 cells was measured in the presence of heated UPM_{10} (H- UPM_{10}) and UPM_{10} . This is the first experimental study on biochemical and pathologic evidences of Beijing dust exacerbating asthma caused by OVA.

Materials and methods

Animals

A total of 96 male ICR mice (5 weeks of age) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). After 1 week of screening out sick mice, mice with abnormal body weight, and mice stressed from different environmental breeding, mice were used at 6 weeks of age. They were fed a commercial diet CE-2 (CLEA Japan, Tokyo, Japan) and given

water *ad libitum*. Mice were housed in plastic cages lined with soft wood chips. The cages were placed in a conventional room, which was air conditioned at 23°C and 55–70% humidity, with a light/dark (12-h/12-h) cycle. The study adhered to the US 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.

Preparation of particles

We have previously reported on the preparation of particles and the characteristics of the chemical composition of the Beijing dust sample used in the present study (Mori et al., 2008). The sample was collected in Beijing in 2005 using a high-volume air sampler with a quartz fiber filter (2500QT-UP; Pallflex). Almost all of the particles in the materials can be classified as particles of 10 μm or less in aerodynamic diameter. The particle size distribution was determined by analyzing a microscopic image using image analysis software (OLS3500/SFT-3500; Olympus and Shimadzu). The lower detection limit of this method was 1 μm . The diameters of 99% of the particles were less than 10 μm , and the volume size distribution derived from the particle size measurements showed that the mode diameter was 7 μm (Mori et al., 2008). The concentrations of chemical elements were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES) (61E Trace and ICP-750; Thermo Jarrell-Ash, USA). The mineral composition of the UPM was analyzed using an X-ray diffractometer (RINT 2000; Rigaku, Japan). Peaks for quartz, feldspar, clay minerals, and gypsum were identified (Mori et al., 2008). Carbon in the material was determined using an elemental analyzer (FLUSH EA 1112; Thermo, USA). UPM were heated at 360°C for 30 min in an electric heater to exclude toxic materials (microbiological materials, sulfate, nitrate, etc.) from adhering to the UPM.

Detection of fungi in particles

The fungi of the UPM was detected by an all-in-one fluorescence microscope (BZ-9000; BIOREVO, Keyence, Japan) and fluorescent dye Fungiflora Y (Technicon International, Tokyo), which specifically bound to β -linked polysaccharides such as β -glucan. This apparatus is a useful tool for demonstrating the fungal cell wall composition (Nishiyama et al., 1995). According to the manufacture's protocol, in brief, approximately 2.5 μg of each particle sample was suspended in 40 μl Fungiflora Y, and after 5 min slides of the particles were completed. Then the slides were observed with the all-in-one fluorescence microscope when the excitation wavelength was 472.5 nm, and the emission wavelength was 520.0 nm.

Analysis of water-soluble components, lipopolysaccharide (LPS), and β -glucan in particles

The concentration of water-soluble components, such as sulfate (SO_4^{2-}) and nitrate (NO_3^-), in the samples was determined using an ion chromatograph (DX-100; Dionex, Sunnyvale, CA, USA) and ICP-AES (61E Trace; Thermo Jarrell-Ash). Each particle sample was measured by kinetic assay using Endospec ES test MK (Seikagaku, Tokyo, Japan) for LPS

activity and by Fungitec G test MK (Seikagaku) for β -glucan activity. According to the manufacture's protocol, in brief, approximately 2.5 mg of each particle sample was suspended in 1 ml water (LPS- and β -glucan-free; Otsuka, Kyoto, Japan) for 1 h and then the sample was allowed to stand on a bench top at room temperature for 2 h. Then the supernatants were recovered and measured for LPS and β -glucan concentration. The limits of detection in the LPS and β -glucan assays were <0.001 EU/ml and 2 pg/ml, respectively.

Study protocol

ICR mice were divided into 6 groups ($n=16$, each group) according to the treatment with particles: normal saline (control), heated UPM (H-UPM), crude UPM, OVA, OVA + H-UPM, and OVA + UPM. These particles were suspended in normal saline (0.9% NaCl) for instillation (Otsuka). This suspension was sonicated for 5 min with an ultrasonic disrupter, UD-201 type with micro tip (Tomy, Tokyo, Japan), under a cooling condition. OVA was dissolved in the same saline. The instillation dose of particles was 0.1 mg per mouse according to previous reports (Ichinose et al., 2008a, 2008b). When the body weight of the mouse was about 36 g, the tidal volume was approximately 0.15 ml and the breathing rate was about 200 breaths per min. The amount of suspended particulate matter (SPM) deposited in the lungs of a single mouse per 2 weeks (90.72 μ g) was calculated using the inhaled value for SPM set by the Chinese national air quality limitation value for SPM (0.15 mg/m³), the tidal volume, and the breathing rate. If 100% of 0.15 mg/m³—which is the Chinese national air quality limitation value for SPM—is accumulated in a lung (90.72 μ g), the instillation dose (0.1 mg/mouse) of particles used in the present study would be 1.1 times the amount accumulated in a lung. In the case of the Human Respiratory Tract Model for Radiological Protection (James et al. 1994), the deposition rate into alveoli is approximately 3% for a 6- μ m diameter particle.

The 0.1 mg particle instillation dose in the present study was approximately 36.8 times the amount of 3% deposition (approximately 2.72 μ g). The instillation dose of OVA was set to 1 μ g per mouse according to a previous report (Hiyoshi et al., 2005). Mice were intratracheally instilled with these particles through a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical, Osaka, Japan) 4 times at 2-week intervals. The control mice were instilled intratracheally with 0.1 ml of normal saline (Otsuka) per mouse. An intratracheal instillation of OVA repeated 4 times at 2-week intervals is needed for the induction of allergic lung inflammation (Ichinose et al., 2008a, 2008b). Therefore, this repeated instillation method was chosen to investigate the aggravating effect of UPM on allergen related-lung eosinophilia. One day after the last intratracheal administration, the mice from all groups were killed by exsanguination under deep anesthesia by intraperitoneal (i.p.) injection of pentobarbital at 12 weeks of age.

Pathological evaluation

Eight of the 16 mice from each group were used for a pathologic examination. The lungs were fixed by 10% neutral phosphate-buffered formalin. After separation of the lobes,

2-mm-thick blocks were taken for paraffin embedding. Embedded blocks were sectioned at a thickness of 3 μ m, and then were stained with hematoxylin and eosin (H&E) to evaluate the degree of infiltration of eosinophils or lymphocytes in the airway from proximal to distal. The sections were also stained with periodic acid-Schiff (PAS) to evaluate the degree of proliferation of goblet cells in the bronchial epithelium. A 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, Tokyo, Japan). The degree of infiltration of eosinophils and lymphocytes in the airway or proliferation of goblet cells in the bronchial epithelium was graded on 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 10% of the airway with eosinophilic inflammatory reaction or with goblet cells stained with PAS; 'mild' as 20–30% of the airway; 'moderate' as 40–50%; 'moderate to marked' as 60–70%; and 'marked' as more than 80% of the airway (Hiyoshi et al., 2005; Ichinose et al., 2006, 2008). Pathological changes were assessed on one slide stained with H&E or PAS per mouse. This evaluation procedure was performed by two pathologists who cross-checked the data in blinded specimens. All values were expressed as mean $\pm$ SD ($n=8$).

Bronchoalveolar lavage

The remaining 8 mice were used for an examination of the free cell contents from bronchoalveolar lavage fluids (BALFs). BALF and cell counts were conducted using a previously reported method (Hiyoshi et al., 2005; Ichinose et al., 2006). In brief, the tracheas were cannulated after the collection of blood. The lungs were lavaged with two injections of 0.8 ml of sterile saline at 37°C by syringe. The lavaged fluid was harvested by gentle aspiration. The average volume retrieved was 90% of the amount instilled (1.6 ml). The fluids from the two lavages were combined, cooled to 4°C, and centrifuged at 1500 rpm for 10 min. The total amount of the lavages collected from individual mice was measured for calculating the protein levels of cytokines and chemokines in BALF. The total cell counts of a fresh fluid specimen were determined using a hemocytometer. Differential cell counts were assessed on cytologic preparations. Slides were prepared using a Cytospin (Sakura, Tokyo, Japan) and stained with Diff-Quik (International Reagents, Kobe, Japan) to identify eosinophils with red granules. A total of 300 cells were counted under oil immersion microscopy. The BALF supernatants were stored at -80°C until it was analyzed for cytokines and chemokines.

Quantitation of cytokines and chemokines in BALF

The cytokine protein levels in the BALF were determined using enzyme-linked immunosorbent assays (ELISA). Interleukin (IL)-5, IL-12, and interferon (IFN)- γ were measured using an ELISA kit from Endogen (Cambridge, MA). Keratinocyte chemoattractant (KC), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , regulated on activation normal T cell expressed and presumably secreted (RANTES), IL-13, IL-4, and eotaxin were measured using an

ELISA kit from R&D Systems (Minneapolis, MN). Monocyte chemotactic protein (MCP)-3 was measured using an ELISA kit from Bender MedSystems (Burlingame, CA).

Antigen-specific IgE and IgG1 antibodies

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

In vitro study for measurement of Toll-like receptors (TLR2 and TLR4)

RAW264.7 cells, which are macrophage-like cells derived from BALB/c male mice, 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 (FBS). They were plated at a concentration of 4 × 10⁵ cells per 60-mm dish, and then UPM with or without heating treatment were added to cells to give a final concentration of 3 µg/ml for 3 h.

Gene expression analysis

Total RNA was extracted by standard procedures using 0.5 ml of Isogen (Nippon Gene) per dish. After DNase treatment of the total RNA, cDNA was synthesized by reverse transcription using Moloney murine leukemia virus (M-MLV). Quantitative polymerase chain reaction (PCR) was performed using an ABI Prism 7000 Sequence Detection System (ABI) under the same conditions as in our previous study (Yoshida et al., 2006). 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 GAPDH. The primers and probes used in this in vitro study were shown in Table 1.

Statistical analysis

Statistical analyses on the pathologic evaluation in the airway, cytokine, and chemokine proteins in BALF were conducted using Tukey for pairwise comparisons (KyPlot ver. 5; Kyens Lab, Tokyo, Japan). Differences among groups were determined as statistically significant at a level of *p* < .05.

Results

The detection of fungi in particles

Figure 1 shows the fluorescence of particles after being stained with Fungiflora Y. There was no fluorescence of H-UPM (Figure 1A and B), but a bright fluorescence was clearly observed in UPM (Figure 1C and D).

Contents of chemical elements, water-soluble components, LPS, and β-glucan in UPM

As shown in Table 2, the oxide compositions in this UPM sample were 32% SiO₂, 9.5% Al₂O₃, 7.2% Fe₂O₃, 1.3% Na₂O,

9.0% CaO, 2.3% MgO, 0.5% TiO₂, 1.7% K₂O, and 12% total carbon. The concentration of SO₄²⁻ was 10,000 µg/g in UPM and nondetected in the H-UPM sample. The concentration of NO₃⁻ was 1900 µg/g in UPM, and also nondetected in the H-UPM sample. The amounts of LPS and β-glucan in the both samples were consistent (Table 3).

Enhancement of pathologic changes in the airway by UPM

Table 4 shows the pathologic changes caused by the Beijing dust in the murine airway, and Figures 2 and 3 illustrate the effects of the Beijing dust on pathological changes in the lungs. No pathologic alterations were found in the lung of the control group (Figures 2A and 3A). No proliferation of goblet cells in the airway was found in the treatment of H-UPM (Figure 2B). Proliferation of epithelial cells in the airway was slight in H-UPM (Figure 3B). UPM alone caused alveolitis, slight bronchitis (Figure 2C), and slight hyperplasia (Figure 3C) of alveolar cells in the airway. The treatment with OVA alone caused slight infiltration of eosinophils in the submucosa (Figure 3D) of the airway, with slight goblet cell proliferation (Figure 2D) in the bronchial epithelium. However, OVA + H-UPM (Figure 2E) and OVA + UPM (Figure 2F) caused increasing proliferation of

Table 1. Primers and probes used in this in vitro study.

Primers and probes	Gene sequence
GAPDH sense	TGCACCACCAACTGCTTAG
GAPDH antisense	GGATGCAGGGATGATGTTTC
GAPDH probe	CAGAAGACTGTGGATGGCCCCTC
TLR2 sense	GAATTGCATCAC CGGTCAGAA
TLR2 antisense	CTGAGCAGA ACAGCGTTTGC
TLR2 probe	CGTCAAATCTCAGAGGATGCTACGAGC
TLR4 sense	AGGACTCTGATCA TGGCACTGTT
TLR4 antisense	GAGTTTCTGATC CATGCATTGG
TLR4 probe	CCAGGAAGCTTGAATCCCTGCATAGAGG

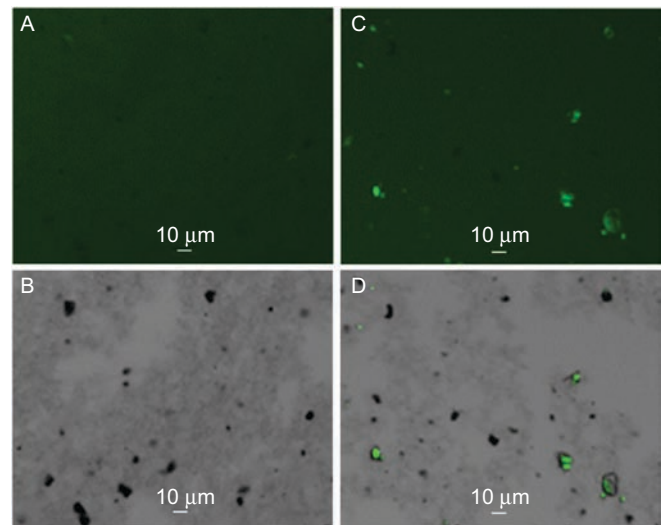


Figure 1. Fluorescent images of the particles stained with Fungiflora Y. Fluorescent images of the particles stained with Fungiflora Y. Bar = 10 µm. (A and B) No fluorescence in UPM heated at 360°C for 30 min. (C and D) Bright fluorescence was seen in crude UPM.

goblet cells ($p < .001$) in the airway compared with the single treatment (Table 4). Both combination treatments caused more prominent infiltration of eosinophils (Figure 2E, F) into connective tissue in the airway. The changes of OVA + H-UPM ($p < .05$) and OVA + UPM ($p < .001$) were significantly different from that of OVA alone (Table 4). The magnitude of eosinophils infiltration was higher in OVA + UPM than in OVA + H-UPM. OVA + H-UPM and OVA + UPM also caused moderate to marked accumulation of lymphocytes in the airway (Figure 3E, F). The degree of the accumulation was higher in OVA + UPM than in OVA + H-UPM (Table 4).

Enhancement of cell numbers in BALF by UPM

Figure 4 shows the cellular profile in BALF. UPM alone increased the number of neutrophils compared with the control, but H-UPM did not. OVA + UPM increased the number of neutrophils compared with the control, UPM alone, and OVA alone, but OVA + H-UPM did not. OVA alone and the

combination treatment with OVA increased the number of macrophages compared with the control. H-UPM, UPM, and OVA alone caused no significant increases of eosinophils compared with the control. The combination treatment with OVA synergistically increased the number of eosinophils compared with the single treatment. OVA + UPM caused a further increase in the number of eosinophils than OVA + H-UPM did. OVA + UPM significantly increased the number of lymphocytes compared with control as well as with OVA and UPM alone.

Enhancement of cytokines and chemokines in BALF by UPM

H-UPM increased protein levels of KC ($p < .05$) in BALF compared with the control (Figure 5). UPM increased KC and MIP-1 α ($p < .05$) compared with the control (Figure 5). The MIP-1 α increased level was constant with increased level of OVA + UPM. However, the increased level of KC was lower in the single treatment groups than that of OVA + UPM. On the other hand, OVA + H-UPM increased KC compared with the control ($p < .001$) and OVA alone ($p < .05$). OVA + UPM increased IL-12 ($p < .05$), KC ($p < .001$), and MIP-1 α ($p < .01$) compared to the control (Figures 5 and 6). OVA + UPM increased KC ($p < .05$) compared with each single treatment, and increased MIP-1 α ($p < .05$) compared with OVA alone. OVA + H-UPM and OVA + UPM synergistically increased protein levels of eosinophil-relevant cytokines, such as IL-4, IL-5, IL-13, MCP-1, and MCP-3, in BALF (Figures 5 and 6) compared with each single treatment. OVA + UPM also increased eosinophil-relevant chemokines, such as eotaxin and RANTES, compared with each single treatment. OVA + UPM caused increases of 1.31-fold in IL-4; 1.46-fold in IL-5; 1.53-fold in IL-13; 2.78-fold in RANTES; 29.8-fold in eotaxin; 1.30-fold in MCP-1; and 2.56-fold in MCP-3 compared with OVA + H-UPM. All levels of increases in the above results were significant.

Table 2. Concentration of chemical elements in UPM.^a

Chemical element compositions	(%)
SiO ₂	32
Al ₂ O ₃	9.5
Fe ₂ O ₃	7.2
Na ₂ O	1.3
CaO	9.0
MgO	2.3
TiO ₂	0.5
K ₂ O	1.7
Total carbon	12
Elemental carbon	7.2
Organic carbon	4.8
Benzo(b)fluoranthene	(10 ppm)
Benzo(e)pyrene	(3 ppm)
Benzo(a)pyrene	(1 ppm)

^aUPM collected from the atmosphere in Beijing.

Table 3. Concentration of water-soluble components, LPS, and β -glucan in UPM.

Particles	SO ₄ ²⁻ (μ g/g)	NO ₃ ⁻ (μ g/g)	Cl ⁻ (μ g/g)	NH ₄ ⁺ (μ g/g)	LPS (EU/mg)	β -Glucan (pg/mg)
UPM ^a	10,000	1900	600	100	0.03	2704
H-UPM ^b	ND	ND	ND	ND	ND	ND

^aUPM: Crude UPM collected from the atmosphere in Beijing, China.

^bH-UPM: UPM were heated at 360°C for 30 min in an electric heater.

ND: not detected.

Table 4. Evaluation of pathological changes in the murine airway.

Group*	Animals (n)	Pathological changes		
		Lymphocytes	Eosinophils	Proliferation of goblet cells
Control	8	0.13 $\pm$ 0.23	0	0.19 $\pm$ 0.26
H-UPM	8	0.19 $\pm$ 0.26	0	0.06 $\pm$ 0.18
UPM	8	0.57 $\pm$ 0.82	0.31 $\pm$ 0.88	0.25 $\pm$ 0.53
OVA	8	1.69 $\pm$ 1.07 [†]	0.88 $\pm$ 0.83	1.56 $\pm$ 1.02 [§]
OVA + H-UPM	8	2.50 $\pm$ 0.65 ^{†¶}	2.06 $\pm$ 0.98 ^{†¶¶}	2.31 $\pm$ 1.13 ^{†¶}
OVA + UPM	8	3.25 $\pm$ 0.60 ^{†‡***}	2.89 $\pm$ 0.83 ^{†‡***}	3.25 $\pm$ 0.60 ^{†‡***}

*Six groups of mice were treated intratracheally with normal saline (control), H-UPM (heated UPM), UPM (crude UPM), ovalbumin (OVA), OVA + H-UPM, and OVA + UPM, 4 times at 2-week intervals. The degree of pathological changes in the airway was estimated: 0 = none; 1 = slight; 2 = mild; 3 = moderate; 4 = moderate to marked; 5 = marked. All values were expressed as mean $\pm$ SD ($n = 8$). Statistical analyses were conducted using Tukey for pairwise comparisons.

[†] $p < .001$ versus control; [‡] $p < .001$ versus OVA; [§] $p < .01$ versus control; [¶] $p < .05$ versus OVA; ^{¶¶} $p < .001$ versus H-UPM; ^{***} $p < .001$ versus UPM.

Enhancement of OVA-specific IgE and IgG1 in serum by UPM

As shown in Figure 7, OVA + H-UPM and OVA + UPM significantly increased the production of OVA-specific IgE as compared with the control and OVA. OVA + UPM markedly elevated OVA-specific IgG1 compared with the control and OVA. These antibodies were higher in the OVA + UPM group than in the OVA + H-UPM group.

In vitro analysis of TLR mRNA expression in RAW264.7 cells

As shown in Figure 8, UPM significantly increased the expression of TLR2 mRNA compared with saline addition, but H-UPM did not. There was no significant difference in the expression of TLR4 mRNA between the control and H-UPM and UPM.

Discussion

Urban PM containing a large amount of constituents, such as elemental carbon, organic carbons (PAHs), and SO_4^{2-} , NO_3^- , derived from air pollutants may cause serious respiratory health problems in humans in Northeast Asia (Chen et al., 2004; Wang et al., 2008). In addition to those produced anthropogenically, the UPM used in this study contained minerals such as SiO_2 (32%) and Al_2O_3 (9.5%), and it also contained microbial materials such as LPS and β -glucan. About half of the UPM in Beijing consists of particles originating from soil. This study demonstrated that H-UPM alone, in which toxic materials (microbiological materials, sulfate, etc.) had been excluded by heating, caused considerably less severe pathological changes. However, whereas crude UPM alone caused bronchitis and alveolitis, and clearly increased

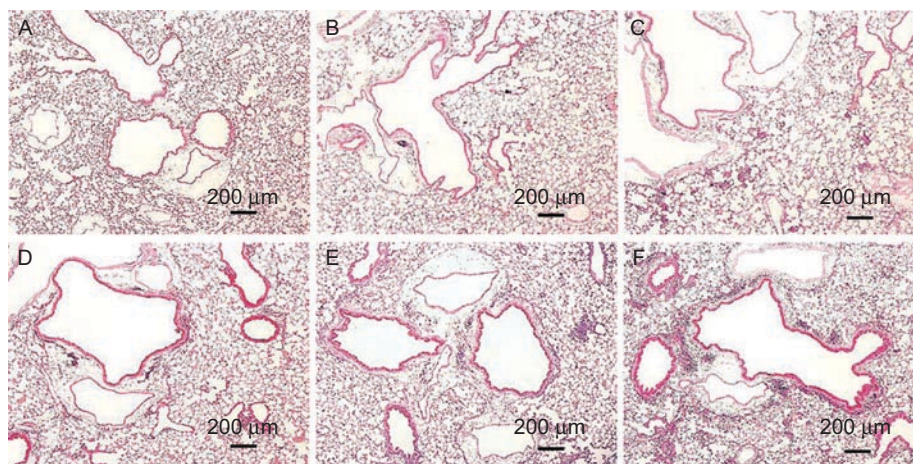


Figure 2. Effects of UPM (with or without heat treatment) on pathological changes in the lungs. (A) No pathological changes in lungs treated with saline. (B) No significant pathological changes in the airway treated with H-UPM. (C) Hyperplasia of alveolar cells and slight alveolitis in the lung treated with UPM. (D) Slight proliferation of goblet cells that have mucus stained pink with PAS solution in the airway epithelium and slight infiltration of inflammatory cells into connective tissue around the airway treated with OVA alone. (E) Moderate proliferation of goblet cells in the airway and moderate infiltration of inflammatory cells into connective tissue around the airway treated with OVA + H-UPM. (F) Marked proliferation of goblet cells and numerous inflammatory cells in the airway treated with OVA + UPM. (A-F) PAS stain; bar = 200 μm .

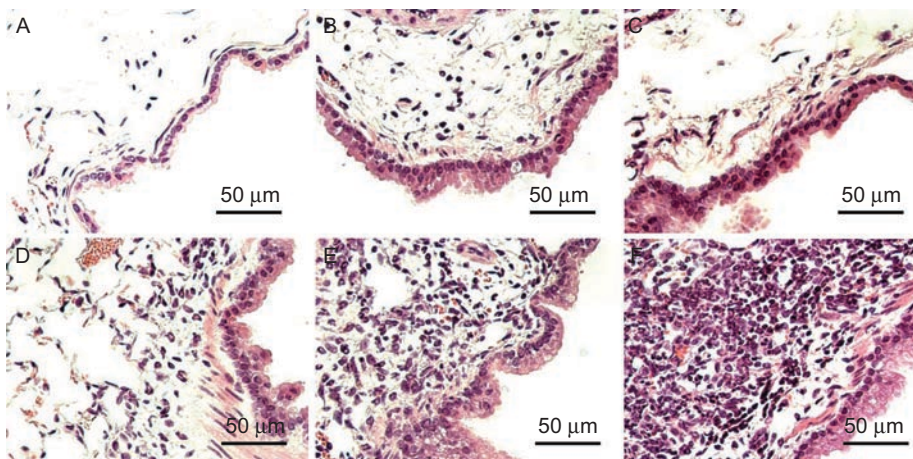


Figure 3. Effects of UPM (with or without heat treatment) on infiltration of inflammatory cells in the airway. (A) No pathological changes treated with saline. (B) Slight proliferation of epithelial cells in the airway treated with H-UPM. (C) Moderate proliferation of epithelial cells treated with UPM. (D) Slight infiltration of eosinophils and lymphocytes into the airway submucosa treated with OVA alone. (E) Moderate infiltration of eosinophils and lymphocytes into connective tissue in the airway treated with OVA + H-UPM. (F) Marked infiltration of eosinophils and lymphocytes into connective tissue in the airway treated with OVA + UPM. (A-F) H&E stain; bar = 50 μm .

neutrophils along with its relevant chemokines, such as MIP-1 α , chemokines such as keratinocyte chemoattractant (KC) elements (Akahoshi et al., 1994), and Th1-relevant cytokine IL-12 (Hsieh et al., 1993) in BALF, H-UPM did not have the same effects. Neutrophilic inflammation accompanying these cytokines and chemokines was induced by LPS (Standiford et al., 1995) or β -glucan (1 $\rightarrow$ 3- β -glucans) (Young et al., 2001). PAHs also could induce these proinflammatory mediators (Goulaouic et al., 2008). Therefore, the microbial materials or PAHs adsorbed onto the UPM may cause, at least partially, neutrophilic inflammation via the expressions of these increased proinflammatory mediators.

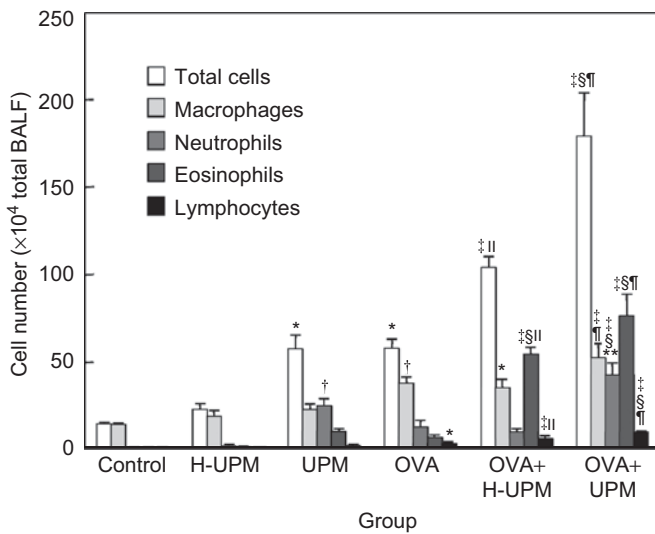


Figure 4. Cellular profile in bronchoalveolar lavage fluids (BALF). All values were expressed as mean $\pm$ SE. * p < 0.05 compared to control; $^{\dagger}p$ < 0.01 compared to control; $^{\ddagger}p$ < 0.001 compared to control; $^{\S}p$ < 0.001 compared to OVA; $^{\parallel}p$ < 0.001 compared to H-UPM; $^{\P}p$ < 0.001 compared to UPM; $^{**}p$ < 0.05 compared to UPM.

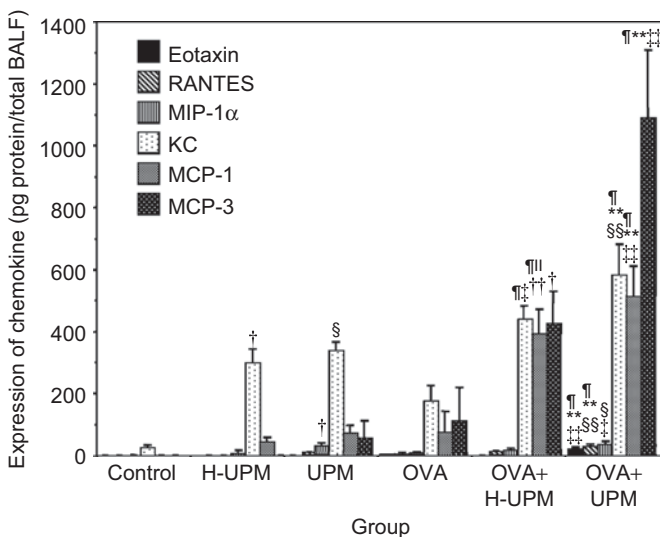


Figure 5. Expression of chemokines in bronchoalveolar lavage fluids (BALFs). All values were expressed as mean $\pm$ SE. $^{\dagger}p$ < .05 versus control; $^{\ddagger}p$ < .05 versus OVA; $^{\S}p$ < .01 versus control; $^{\parallel}p$ < .01 versus OVA; $^{\P}p$ < .001 versus control; $^{**}p$ < .001 versus OVA; $^{**}p$ < .01 versus H-UPM; $^{**}p$ < .001 versus UPM; $^{**}p$ < .01 versus UPM.

Crude UPM enhanced allergic airway inflammation and the proliferation of goblet cells in the airway epithelium in OVA-stimulated mice, which was evidenced by cellular profiles of BALF and histological examinations. The exacerbated antigen-related airway inflammation was concomitant with amplified expression of proinflammatory molecules such as eotaxin, IL-5, and MCP-3 in BALF as well as the production of antigen-specific antibodies. The enhancing effects were more remarkable in the OVA + UPM group than in the other groups. OVA causes a slight proliferation of goblet cells and an infiltration of eosinophils and lymphocytes in the airway, which are the pathology correlatives seen in human asthma. The toxic released mediators from eosinophils, such as platelet-activating factors, reactive oxygen radicals, leukotrienes, and major basic proteins, play an important role in inducing bronchial mucosal injury (Gleich, 1990). Eosinophils also cause bronchial smooth muscle cell death in asthma (Solarewicz-Madejek et al., 2009). The transformation of airway epithelial cells to goblet cells by OVA in the crude UPM group and the H-UPM group correlated with the amount of eosinophils introduced into the submucosa of the airway. Therefore, the injury to airway epithelial cells along with the proliferation of goblet cells may be induced by the activated eosinophils. The aggravating effects of UPM were greater than those in H-UPM.

As one of the organic substances excluded by heating, a large amount of sulfate (SO_4^{2-}) produced by the sulfur oxide (SO_2) in air pollutants was contained in the crude UPM used in the present study. Previous reports have shown that both Asian sand dust (ASD) and ASD + sulfate (SO_4^{2-}) enhanced eosinophils recruitment induced by OVA in the alveoli and in the submucosa of the airway. However, a further increase in eosinophils by the addition of SO_4^{2-} was not observed (Hiyoshi et al., 2005). We concluded that extra sulfate did not contribute to the increase of eosinophils in the airway and BALF (Hiyoshi et al., 2005). Therefore, it seems that sulfate of

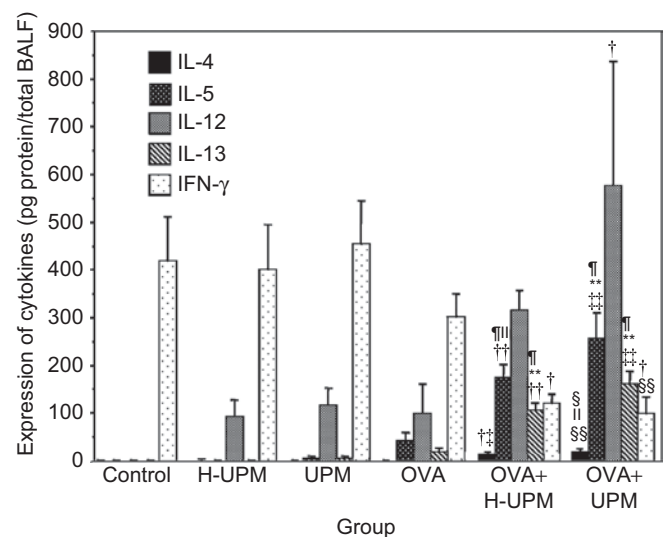


Figure 6. Expression of cytokines in bronchoalveolar lavage fluids (BALF). All values were expressed as mean $\pm$ SE. $^{\dagger}p$ < .05 versus control; $^{\ddagger}p$ < .05 versus OVA; $^{\S}p$ < .01 versus control; $^{\parallel}p$ < .01 versus OVA; $^{\P}p$ < .001 versus control; $^{**}p$ < .001 versus OVA; $^{**}p$ < .001 versus H-UPM; $^{**}p$ < .001 versus UPM; $^{**}p$ < .01 versus UPM.

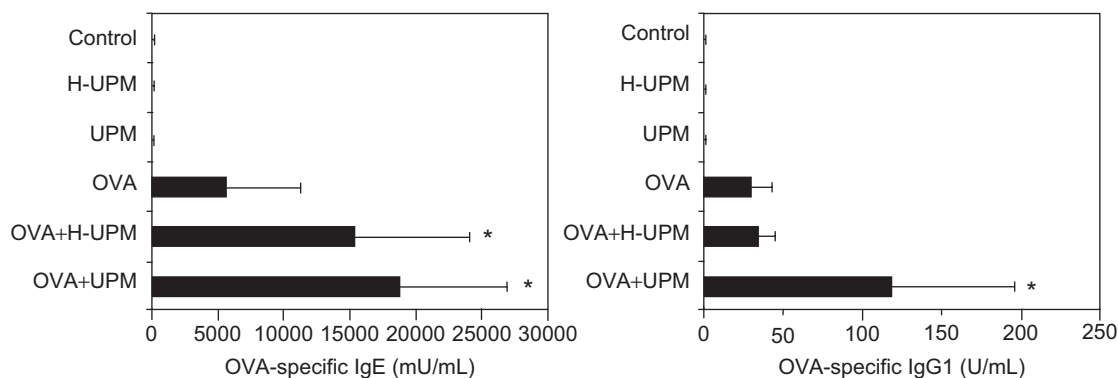


Figure 7. Effect of UPM (with or without heat treatment) on OVA-specific IgE and IgG1 production in serum. According to the manufacturer's protocol, 1 U of the anti-OVA IgE is defined as 1.3 ng of the antibody, and 1 U of the anti-OVA IgG1 as 160 ng of the antibody. Results are expressed as mean $\pm$ SE. The asterisk indicates significant at $p < .05$ compared to control, H-UPM, UPM, and OVA.

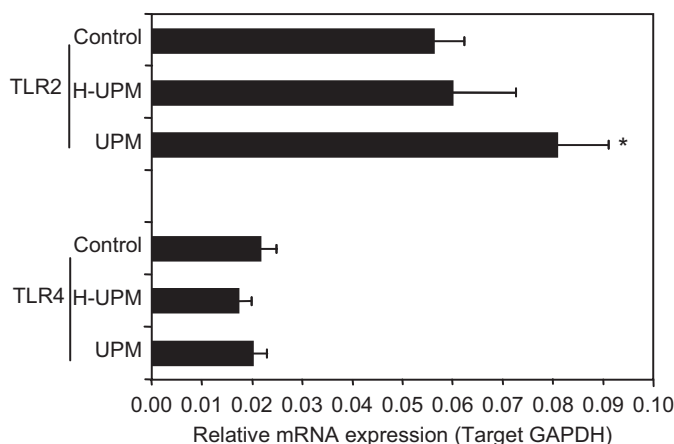


Figure 8. Effects of UPM (with or without heat treatment) on TLR2 and TLR4 mRNAs (relative to GAPDH mRNA) in RAW264.7 mouse macrophage cells. Results are expressed as mean $\pm$ SE. The asterisk indicates significant at $p < .05$ compared to control and H-UPM.

air pollutant origin is not involved in the aggravating effects of lung eosinophilia and allergy diseases.

On the other hand, toxic organic substances, such as LPS and β -glucan, were adsorbed onto the UPM. LPS is a major constituent of the outer cell wall of gram-negative bacteria and the predominant inducer of inflammatory responses to these bacteria (Beutler et al., 2002; Knapp et al., 2006; Opal et al., 2003). β -Glucan is a unique component of the fungal cell wall (Hearn and Sietsma, 1994). The reduced effects of H-UPM on the pathological changes could be due to the exclusion of the toxic organic substances by heat treatment. Therefore, it seems that microbial materials adsorbed on UPM contribute to the development of bronchitis and alveolitis.

Toll-like receptor (TLR) 2 is a receptor for zymosan (β -glucans), peptidoglycan of gram-positive bacteria (Schwandner et al., 1999), and TLR4 is a receptor for LPS of gram-negative bacteria (Beutler, 2002). TLR2 ligands can activate an OVA-associated Th2-biased immune response, such as the induction of Th2-associated effector molecules such as IL-13, IL-5, and OVA-specific IgG1 and IgE. TLR2 ligand can have an effect on the development of Th2-associated diseases such as experimental asthma (Redecke et al., 2004). In

our previous study, ASD aggravated OVA-induced lung eosinophilia. We suggested that the aggravated lung eosinophilia was due to the activation of a Th2-associated immune response via the activation of TLR2 (but not TLR4) by microbial components adhering to ASD (Ichinose et al., 2008a). The UPM tested in this study increased the expression of TLR2 mRNA. Therefore, microbial materials adsorbed onto UPM may contribute to the enhancement of lung eosinophilia related to allergen. This UPM might contain diesel exhaust particles (DEPs) emitted from diesel-powered automobiles. DEP, extract of organic chemicals (EOCs) from DEPs, and the polycyclic aromatic hydrocarbons (PAHs) in the EOCs have ability to exacerbate antigen-related lung eosinophilia with the adjuvant effects of antigen-specific immune antibodies (Stevens et al., 2009; Takano et al., 1997; Yanagisawa et al., 2006).

The major chemical inorganic elements in the UPM tested in this study were elemental carbon and silica (SiO_2), which were derived mainly from quartz (Mori et al., 2008). Quartz, amorphous or crystalline silica, have been reported to cause inflammatory responses with the release of inflammatory cytokines in the lungs of rats (Ernst et al., 2002; Murphy et al., 1998). Previous studies have reported that crystalline silica (standard quartz DQ12 with particle size less than 5 microns) or amorphous silica (Mancino et al., 1980, 1984) was able to stimulate in mice the production of IgE and IgG1 antibodies to a single 1- μg dose of ovalbumin. On the other hand, elemental carbon, such as carbon black (particle sizes, 14 and 50 nm) and DEPs—after extracted with dichloromethane (washed DEPs)—also can exacerbate lung eosinophilia with an increased expression of proinflammatory molecules as well as an elevated concentration of OVA-specific IgE antibody (Inoue et al., 2005; Yanagisawa et al., 2006). Therefore, the deterioration of the allergic inflammation along with the elevation of the OVA-specific IgE antibody by heated UPM might be due to the presence of those elemental particles.

Eosinophils and Th2 lymphocytes and their released inflammatory mediators play a crucial role in allergic asthma (Pearlman, 1999). H-UPM and UPM alone showed no increase of asthma-related mediators in BALF. However, OVA + H-UPM and OVA + UPM caused synergistic increases of eosinophil-relevant cytokines and chemokines, such as IL-5 (Foster et al.,

1996; Robinson et al., 1993), MCP-1 (Dunzendorfer et al., 2001), MCP-3 (Nagase et al., 1999), and RANTES (Beck et al., 1997), and Th2 cytokines IL-4 (Mosmann et al., 1989) and IL-13 (D'Andrea et al., 1995) in BALF, compared with H-UPM, UPM, or OVA alone. Eotaxin, which is a major chemoattractant for eosinophils (Rothenberg et al., 1997), only increased in the OVA + UPM group. The expressions of these cytokines and chemokines in OVA + UPM were much greater than those in OVA + H-UPM, suggesting that organic substances, such as microbial materials or chemical substances, excluded by heating are at least partly related to the enhancement of eosinophil recruitment via these chemical mediators in the alveoli and airway. Thus, the changes of the biological components in BALF were well consistent with the pathological changes in the lungs. On the other hand, the combination of OVA with H-UPM or UPM suppressed the expression of Th-1 cytokine like IFN- γ in the BALF. Interleukin-4, IL-10, and IL-13 as well as IL-5 released from Th-2 lymphocytes are well known to suppress IFN- γ or TNF- α (D'Andrea et al., 1993, 1995). The suppression of IFN- γ could be due to synergistically increased Th-2 cytokines.

In the present study, an adjuvant effect of UPM toward the OVA-specific IgE and IgG1 production was detected. Therefore, the antibodies may play an important role in the aggravation of allergic inflammation caused by the UPM, which was tested in the present study.

An earlier study has shown that PM_{2.5} is more closely associated than PM₁₀ with both acute and chronic respiratory effects, including bronchial asthma and subsequent mortality (Peters et al., 1997). Therefore, a comparative investigation of the effects of the different particle sizes (PM_{2.5} and PM₁₀) in UPM on the time course of allergen-related inflammatory responses, the protein expression of proinflammatory cytokines and chemokines, and their gene expressions seems to be necessary in the next stage of study. In addition, it is necessary to conduct a future study of the comparative effects of UPM collected in the USA and Europe.

Air pollution over Northeast Asia has become more severe in the last 20 years. Suspended particulate concentration is at an especially high level in Beijing. The UPM tested in this study contained a large amount of β -glucan derived from fungi, which might be derived from dust ascending during the Beijing construction boom. Biogenic particles such as bacteria, fungi, virus, pollen, cell debris, etc., are also transported long distances together with materials produced by the anthropogenic pollution of fossil fuel combustion and with Asian sand dust. The hazardous effects of bioaerosols (biogenic particles) on human respiratory disease are also becoming a public concern.

Conclusions

Our findings suggest that UPM aggravates lung eosinophilia, possibly via activation of the Th2-associated immune response. The microbial materials and chemical materials of air pollutant origin contained in UPM may be partly responsible for allergen-induced lung eosinophilia. Atmospheric exposure to chemical products from fossil fuel combustion,

fungi, bacteria, and silica-carrying particulate matters may affect human health directly through allergic induction of respiratory stress. The experimental findings in the present study may serve as a warning about the ill effects of UPM and wind-borne sand dust with UPM on the human respiratory system in Northeast Asia.

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Declaration of interest

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