Effects of Fetal Exposure to Urban Particulate Matter on the Immune System of Male Mouse Offspring

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Urban particulate matter (UPM) has been shown to have an aggravating effect on Th2-associated immune systems in adult mice. However, the effects of fetal exposure to UPM on immune response in offspring have not been elucidated. In the present study, we administered UPM (200 µg/animal) by intratracheal injection to pregnant dams on days 7 and 14 of gestation. Subsequently, 9- and 24-week-old male offspring were intratracheally injected with ovalbumin (OVA) (four times at 2-week intervals) to create a mouse model of bronchial asthma. We then evaluated the progression of allergic manifestations in the offspring through histological findings, the number of inflammatory cells in bronchoalveolar lavage fluid (BALF), and protein concentration of cytokines and chemokines in BALF 5, 10, 15, and 30 weeks after birth. Histological examination showed that fetal exposure to UPM alone caused slight eosinophil and lymphocyte infiltration in the submucosa of the airway and bronchial epithelium and significant increases in the number of macrophages. Moreover, postnatal intratracheal administration of OVA to offspring exposed to UPM *in utero* caused significant increases in the numbers of macrophages, eosinophils, and lymphocytes and in the concentrations of their relevant cytokines and chemokines, showing that fetal exposure to UPM aggravated the chemically sensitized immune system of male offspring.

Key words immune system; next generation; particulate matter; environmental factor; cytokine; lymphocyte

Ambient air pollution has been extensively shown to affect human health.^{1–3} Numerous epidemiological studies have demonstrated that exposure to urban particulate matter (UPM) air pollution, which occurs in many urban and industrial environments, is associated with an increase in childhood health risks, such as respiratory symptoms and hospital admissions for asthma.^{4–6} Several recent studies have documented the adverse effects of UPM on respiratory disease in children,⁷ mortality rates from reduced cardiopulmonary function, lung cancer incidence,³ the number of low birth weight infants,⁸ and asthma.⁹ In addition, the causes of childhood allergies, the incidence of which has increased in recent years, are related to dietary and residential environmental factors. Lifestyle environmental factors (such as environmental tobacco smoke and residential location) that affect pregnant women are also believed to be involved, although their mechanisms of action remain unknown.¹⁰ In this regard, the effects of fetal exposure during pregnancy of women smokers include increases in the risk of intrauterine growth retardation and incidence of childhood bronchial asthma.¹¹ Exposure of pregnant female mice to diesel exhaust particles (DEP) can cause increased allergic susceptibility in offspring,¹² and exposure of adult mice to dioxin, which is found in trace amounts in DEP, can decrease antibody production.¹³ However, the effects of ambient particulate matter inhaled by pregnant women on the health of their offspring remains to be elucidated; therefore, such an investigation is important.

Therefore, in this study, we designed experiments to simulate immune responses in offspring of pregnant women exposed to UPM. We combined exposure of pregnant ICR mice to UPM with postnatal administration of ovalbumin (OVA) to their male offspring to determine whether *in utero* exposure

to UPM aggravates the postnatally sensitized immune system. We proposed the following hypotheses. *In utero* exposure to UPM: (1) aggravates the sensitized immune system; (2) influences lung histopathology following OVA sensitization; and (3) promotes cytokine and chemokine production in OVA-exposed mice.

To test these hypotheses, we created a mouse model of bronchial asthma by exposing male offspring to OVA and then sequentially evaluated the effects of fetal exposure to UPM on their allergic manifestations.

MATERIALS AND METHODS

Animals Forty pregnant ICR mice purchased from CLEA Japan (Tokyo) were used [induction at day 5 post-coitum (p.c.; the day the plug was found was considered to be day 0 p.c.)]. Mice were divided into two groups of 20: the UPM and control groups. Up to day 16 of induction, the mice were housed five per cage, and each mouse was caged separately from day 17 of induction. The mice were kept under the following conditions: temperature, 23–25°C; humidity, 50–70%; and 12-h light/dark cycle. The mice had free access to food (CE-2 solid food; CLEA, Japan) and water. The study was conducted in accordance with the Laboratory Animal Facility Usage Guidelines compiled by the Oita University of Nursing and Health Science (approval number, 310, 2009).

Preparation of Particles Preparation of particles and the characteristics of the chemical composition of the Beijing dust sample used in the present study have been reported previously.¹⁴ The sample was collected in Beijing in 2005 using a high-volume air sampler with a quartz fiber filter (2500QT-UP; Pallflex). Almost all particles in the materials were ≤10 µm in aerodynamic diameter. Particle size distribution was determined by analyzing a microscopic image using image analysis

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Fig. 1. Experimental Time Frame for Prenatal and Postnatal Treatments and Euthanasia

Times are expressed relative to the birth of offspring. GD, gestation day.

software (OLS3500/SFT-3500; Olympus and Shimadzu, Japan). The lower detection limit of this method was $1\mu\text{m}$. The diameters of 99% particles were $<10\mu\text{m}$, and the volume size distribution derived from the particle size measurements showed that the mode diameter was $7\mu\text{m}$.¹⁴ The concentrations of chemical elements were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, 61E Trace and ICP-750; Thermo, Jarrell-Ash, U.S.A.). The mineral composition of UPM was analyzed using an X-ray diffractometer (RINT 2000; Rigaku Corporation, Japan), and peaks were identified for quartz, feldspar, clay minerals, and gypsum.¹⁴ UPM used in this study contained several elements such as Si (14.9%), S (3.91%), P (0.145%), Cl (0.87%), Ca (6.69%), Al (5.04%), Fe (2.92%), K (1.37%), Mn (686mg/kg), Ba (874mg/kg), Sr (469mg/kg), Cu (104mg/kg), and As (90mg/kg) and it also contained microbial materials such as lipopolysaccharide (LPS) and β -glucan.¹⁴ Carbon in the material was determined using an elemental analyzer (Flush EA 1112; Thermo). UPM was heated at 360°C for 30min in an electric heater to eliminate toxic materials (microbiological materials, sulfate, nitrate, etc.).

Particle Administration UPM was suspended in normal saline (0.9% NaCl) for instillation (Otsuka Co., Kyoto, Japan). This suspension was sonicated for 5min using an ultrasonic disrupter (UD-201 type with micro tip; Tomy, Tokyo, Japan) under cooling conditions. The instillation dose of particles was $200\mu\text{g}/\text{mouse}$; the dose was determined in a previous report.¹⁵ The particles were intratracheally administered to pregnant mice on days 7 and 14 of gestation. Exposure began after implantation and initial organ development had taken place. The interleukin (IL)-7 receptor (IL-7R) plays an essential role in the formation of T cells. The IL-7R cells in the Lin⁻ c-kit⁺ population (T-cell lineage-restricted progenitors/natural killer cells/dendritic cells) circulate exclusively between days 11

and 14 of gestation.¹⁶ The amount of UPM deposited in the lungs of a single mouse every 2 weeks ($90.72\mu\text{g}$) was calculated using the value set by the Chinese national air quality limitation for inhaled UPM ($150\mu\text{g}/\text{m}^3$), the tidal volume, and the breathing rate. On the premise that 100% of $150\mu\text{g}/\text{m}^3$ is accumulated in a lung ($90.72\mu\text{g}$), the instillation dose ($200\mu\text{g}/\text{mouse}$) of particles used in the present study was 4.4 times that amount.

Offspring Mice that were delivered after an average gestation of $18\pm 0.5\text{d}$ were used. At the age of 12d, six males (from each dam) were selected, weaned at 25–26d after birth, and housed eight per cage.

Study Protocol Male ICR mouse offspring were divided into 12 groups (16 per group) according to the treatment used: normal saline (control: 5, 10, 15, and 30 weeks; OVA: 15 and 30 weeks) and fetal exposure to UPM (UPM: 5, 10, 15, and 30 weeks; UPM+OVA: 15 and 30 weeks). OVA was dissolved in the same saline as the particles, and the instillation dose was set at $1\mu\text{g}/\text{mouse}$ according to a previous report.¹⁷ Four intratracheal instillations of OVA at 2-week intervals are needed to induce allergic lung inflammation.¹⁸ Therefore, this method was chosen to investigate the aggravating effect of fetal exposure to UPM on allergen-related lung eosinophilia. One day after the last intratracheal administration, mice from all groups were killed by exsanguination under deep anesthesia

Table 1. Effects of UPM Exposure on Dams and Fertility

	Control	UPM
Fertility	100%	90%
Gestation length (d)	18.6 ± 0.4	18.8 ± 0.3
Litter size	13.1 ± 4.4	13.7 ± 3.6
Male: female ratio	0.57 ± 0.15	0.53 ± 0.10

Results are mean $\pm$ S.D.

Fig. 2. Pathological Changes in the Lungs after of Fetal Exposure to Urban Particulate Matter (UPM)

A–D: 15-week-old offspring. E–H: 30-week-old offspring. A and E: controls; no pathological changes. B and F: UPM; moderate infiltration of inflammatory cells. C and G: ovalbumin (OVA); slight infiltration of inflammatory cells. D and H: UPM+OVA; marked infiltration of inflammatory cells into connective tissue around the airway. Hematoxylin and eosin (HE) staining. Original magnification $\times 100$.

Fig. 3. Cellular Profiles in Bronchoalveolar Lavage Fluid (BALF)

A: 5-week-old offspring. B: 10-week-old offspring. C: 15-week-old offspring. D: 30-week-old offspring. All values were expressed as mean $\pm$ standard error (S.E.). * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control; *** $p < 0.001$ vs. control; # $p < 0.05$ vs. urban particulate matter (UPM); ## $p < 0.01$ vs. UPM; ### $p < 0.001$ vs. UPM; \$ $p < 0.05$ vs. ovalbumin (OVA); \$\$ $p < 0.01$ vs. OVA; \$\$\$ $p < 0.001$ vs. OVA. ■: total cells; ■: macrophages; ■: neutrophils; ■: eosinophils; ■: lymphocytes.

with an intraperitoneal injection of pentobarbital at 5, 10, 15, and 30 weeks of age (Fig. 1).

Pathological Evaluation Eight mice from each group were used for pathological examination. The lungs were fixed in 10% neutral phosphate-buffered formalin. After separation of the lobes, 2-mm blocks were taken for paraffin embedding. Embedded blocks were sectioned at a thickness of $3\mu\text{m}$ and then stained with hematoxylin and eosin (HE). Pathological

analysis of the inflammatory cells and epithelial cells in the airway of each lung lobe was performed on the slides using a Nikon Eclipse light microscope (Nikon Co., Tokyo, Japan).

Bronchoalveolar Lavage The remaining eight mice from each group were used to examine the free cell contents from bronchoalveolar lavage fluid (BALF). BALF and cell counts were performed using a method reported previously.^{17,19} In brief, the tracheas were cannulated after blood collection. The

lungs were lavaged with two injections of 0.8 mL sterile saline at 37°C. 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 lavages collected from individual mice was measured to calculate cytokine and chemokine levels in BALF. The total cell counts of a fresh fluid specimen were determined using a hemocytometer. Differential cell counts were assessed on cytological preparations. Slides were prepared using Cytospin (Sakura Co., Ltd., Tokyo, Japan) and stained with Diff-Quik (International Reagents Co., 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 analysis for cytokines and chemokines.

Quantitation of Cytokines and Chemokines in BALF

Cytokine levels in BALF were determined using enzyme-linked immunosorbent assays (ELISA). IL-5, IL-12, and tumor necrosis factor (TNF) were measured using an ELISA kit from Endogen, Inc. (Cambridge, MA, U.S.A.). Keratinocyte chemoattractant (KC), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , regulated on activation, normal T cell expressed and secreted (RANTES), IL-13, IL-4, and eotaxin were measured using an ELISA kit from R&D Systems Inc. (Minneapolis, MN, U.S.A.). MCP-3 was measured using an ELISA kit from Bender MedSystems Inc. (Burlingame, CA, U.S.A.).

Statistical Analysis Using KyPlot version 5 (Kyens Lab Inc.), the Tukey test for pairwise comparisons was used for statistical analysis. A p value of <0.05 was considered to represent statistical significance.

RESULTS

Effects of UPM Administration on Maternal and Fetal Growth The effects of UPM administration on dams were determined by comparing length of gestation, litter size, fertility, and gender ratio between the control and UPM groups, and no significant differences were observed in these parameters between the two groups (Table 1).

Pathological Changes in Lungs Figure 2 illustrates the effects of fetal exposure to UPM on pathological changes in the lungs at 15 and 30 weeks of age. Fetal exposure to UPM caused a slight eosinophil and lymphocyte infiltration in the submucosa of the airway and in the bronchial epithelium (Figs. 2B,F), which was not observed in the control group (Figs. 2A,E). In the mouse model of bronchial asthma created by exposure to OVA, eosinophil and lymphocyte infiltration in the bronchial submucosa was also observed (Figs. 2C,G). In the UPM+OVA group, marked eosinophil, neutrophil, and lymphocyte infiltration was observed (Figs. 2D,H).

Effects on the Number of Inflammatory Cells in BALF

To evaluate the effects of fetal exposure to UPM on BALF, we analyzed each type of inflammatory cell individually. The total number of cells in BALF was 2.0 and 2.4 times higher, respectively, in the UPM group at 5 and 30 weeks of age than in the control group ($p<0.001$ and $p<0.05$, respectively) (Figs. 3A,D). This number was 2.0 times higher in the UPM+OVA group at 15 weeks than in the OVA group ($p<0.05$) (Fig. 3C). The number of macrophages in BALF was 1.8 and 1.6

times higher in the UPM group at 5 and 30 weeks, respectively ($p<0.001$ and $p<0.05$, respectively) (Figs. 3A,D) than in the control group. This number was 1.6 times higher in the UPM+OVA group at 15 weeks than in the OVA group ($p<0.05$) (Fig. 3C). Fetal exposure to UPM induced neutrophils in BALF at 10 weeks and eosinophils in BALF at 5, 10, and 30 weeks (Figs. 3A, B, D). At 30 weeks of age, the number of lymphocytes in BALF was significantly higher in the UPM group compared with the control group and in the UPM+OVA group compared with the OVA group ($p<0.01$) (Fig. 3D).

Effects on Cytokine and Chemokine Levels in BALF

At age 15 weeks, fetal exposure to UPM induced no significant changes in cytokine and chemokine levels in BALF (Tables 2 and 3).

At 30 weeks, UPM+OVA significantly increased the levels of IL-5 ($p<0.05$), IL-13 ($p<0.05$), MCP-1 ($p<0.05$), and RANTES ($p<0.05$) compared with OVA alone, and significantly increased the levels of IL-5 ($p<0.05$), IL-12 ($p<0.001$), IL-13 ($p<0.01$), KC ($p<0.001$), MCP-1 ($p<0.05$), and RANTES ($p<0.001$) compared with UPM alone.

DISCUSSION

Recently, the prevalence of childhood allergies has been increasing, the causes of which are believed to include changes in dietary habits and residential environments.²⁰ Lifestyle environmental factors such as exposure of pregnant women to particulate matter are also thought to be a cause, but the mechanisms of action have not been elucidated. Therefore, in this study, we administered UPM collected from the atmosphere to pregnant mice and evaluated various effects on the immune system of their offspring. We previously reported that exposure of adult mice to microparticles, including urban particulate matter and carbon black particles, caused neutrophil infiltration in lung tissue as well as marked increase in the number of eosinophils in BALF related to administration of OVA.^{17,21}

In the present study, we compared lung histology in groups of mice exposed *in utero* to UPM (UPM group, UPM+OVA group) with that in the respective control groups (untreated group, OVA group) and found marked infiltration of inflammatory cells such as eosinophils in the submucosa of the airway in the UPM+OVA group. This suggests that inflammation is caused by fetal exposure to UPM in the absence of direct inhalation and that this process continues after birth. Although OVA-induced inflammatory cell counts in BALF were increased by fetal exposure to UPM, the increase was smaller in younger groups of mice (5–10 weeks) than in adult mice (30 weeks). Moreover, the number of macrophages was increased by fetal exposure to UPM (at 5, 15, and 30 weeks in the UPM and UPM+OVA groups), indicating enhancement of antigen presentation that might lead to later Th2-associated immune responses.

This increase in macrophages in mouse offspring exposed to UPM *in utero* may be related to blood cell differentiation during the fetal period, because maternal exposure could prime the fetal immune system, and subsequent immunization during the postnatal period may increase neonatal immunity.²² Furthermore, UPM+OVA increased the number of eosinophils in BALF, which suggests the possible aggravation

Table 2. Concentration of Cytokines in Bronchoalveolar Lavage Fluid

	Cytokine expression (pg protein/total BAL supernatant)				
	IL-4	IL-5	IL-12	IL-13	TNF
15 weeks					
Control	0	0	70.0±27.4	0	0
UPM	0	0	44.4±21.9	0	0
OVA	0	29.6±20.1	121.6±54.2	22.4±16.7	0
UPM+OVA	0	52.8±36.0	187.0±116.7	14.7±14.7	0
30 weeks					
Control	14.3±6.9	0	0	0	17.3±7.2
UPM	6.3±4.2	0	30.3±21.3	0	50.3±10.5
OVA	9.6±6.9	10.6±3.5	188.4±29.4**.#	7.1±1.8	11.2±11.2
UPM+OVA	9.5±4.9	131.4±58.6*.,#,\$	277.2±64.7***####	24.7±8.9**.,#,\$	19.9±10.6

Bronchoalveolar lavage (BAL) was performed 24h after the last intratracheal instillation of OVA. All values were expressed as mean±S.E. (n=8). **p*<0.05 vs. Control, ***p*<0.01 vs. Control, ****p*<0.001 vs. Control. #*p*<0.05 vs. UPM, .*p*<0.01 vs. UPM, .*p*<0.001 vs. UPM, \$*p*<0.05 vs. OVA.

Table 3. Concentration of Chemokine in Bronchoalveolar Lavage Fluid

	Chemokine expression (pg protein/total BAL supernatant)					
	Eotaxin	KC	MCP-1	MCP-3	MIP-1α	RANTES
15 weeks						
Control	0	0	2.4±0.1	0	0	1.4±0.5
UPM	0	0	2.4±0.2	0	0	1.8±0.8
OVA	2.8±2.8	31.1±23.5	9.8±2.9	32.2±32.2	0	2.5±1.1
UPM+OVA	4.7±4.7	52.0±27.2	14.7±7.0	5.4±5.4	1.4±1.4	3.0±1.5
30 weeks						
Control	0	3.9±3.9	0	0	0	0
UPM	0	0	0	0	0	0
OVA	0	91.2±16.8	4.6±4.6	0	10.4±5.5	74.7±24.6
UPM+OVA	20.2±15.7	116.3±20.5***.,###	117.7±52.5*.,#,\$	118.4±75.7	9.5±5.3	329.6±102.9***.,###,\$

Bronchoalveolar lavage (BAL) was performed 24h after the last intratracheal instillation of OVA. All values were expressed as mean±S.E. (n=8). **p*<0.05 vs. Control, ****p*<0.001 vs. Control. #*p*<0.05 vs. UPM, .*p*<0.01 vs. UPM, .*p*<0.001 vs. UPM, \$*p*<0.05 vs. OVA.

of type I allergic asthma by fetal exposure to UPM. This result is also consistent with lung histological analysis. From these finding, we speculate that with regard to the prevalence of childhood allergies and aggravation of asthma, the living environment of pregnant women, including smoking and exposure to pollutants such as UPM, could affect the immune system of their offspring.

The various biological components in BALF may reflect an event in the lungs. Fetal exposure to UPM increased the number of macrophages, eosinophils, and lymphocytes as well as the levels of their relevant cytokines and chemokines, such as IL-5,²³⁾ IL-13,²⁴⁾ MCP-1,²⁵⁾ and RANTES,²⁶⁾ induced by administration of OVA. Because RANTES is known to be strongly involved in macrophage enhancement as well as eosinophil migration and eosinophil activation, the increase in the number of macrophages and eosinophils in our study may be partly related to the higher levels of RANTES. However, there was no correlation between the number of several cell types or the concentration of cytokines and chemokines in the OVA group at 15 and 30 weeks, and other factors may contribute to the increase in cell number. Our results suggest that fetal exposure to UPM and postnatal intratracheal administration of OVA enhance allergic lung inflammation by activating the Th2-associated immune response through increase in cytokines and chemokines (such as IL-5, IL-13, MCP-1, and RANTES), which play an important role in bronchial

asthma.^{27,28)}

Smoking in pregnant mothers, by virtue of indirect fetal exposure, can cause and increase the risk of intrauterine growth retardation, increase the incidence of asthma in children after birth^{11,29,30)} and affect pulmonary function in adulthood ("fetal origin hypothesis").³¹⁾ Thus, predispositions to health problems and disease may be formed very early in life. As shown in this study, the maternal environment and exposure to UPM *in utero* may affect the immune system of offspring. The particles of approximately 240nm diameter did not penetrate the blood-placenta barrier.³²⁾ Therefore, we used UPM with a mode diameter of 7μm¹⁴⁾ to study the indirect effects of fetal exposure to UPM on male offspring.

In this study, we found that fetal exposure to UPM: 1) caused an increase in the number of macrophages in BALF of offspring (UPM group); 2) caused an increase in the number of eosinophils, which regulate the immune system, induced by OVA (UPM+OVA group); and 3) induced the expression of IL-5, IL-13, MCP-1, and RANTES, which aggravate allergic inflammation (UPM+OVA group). Our findings suggest that the inhalation of UPM by pregnant women has various effects on the fetus and may be involved in the increased prevalence of childhood allergies.

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