Asian Sand Dust Enhances Allergen–Induced Th2 Allergic Inflammatory Changes and Mucin Production in BALB/c Mouse Lungs

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Purpose: Recent studies have reported that Asian sand dust (ASD) has a potential risk of aggravating airway inflammation. The purpose of this study was to investigate the effect of ASD on inflammation and mucin production in the airways of allergic mice. Methods: Forty BALB/c female mice were divided into four groups: saline (group 1); ASD (group 2); ovalbumin (OVA) alone (group 3); and OVA+ASD (group 4). OVA-specific immunoglobulin E (IgE) in serum and interleukin (IL)-4, IL-5, IL-13, and interferon-γ (IFN-γ) in bronchoalveolar lavage fluid (BALF) were measured by enzyme-linked immunosorbent assay (ELISA). Hematoxylin & eosin (H&E) and Periodic acid-Schiff (PAS) staining was performed on lung tissues. In addition, immunohistochemical staining for IL-4, IL-5, MUC5AC, and transforming growth factor alpha (TGF-α) was conducted. Results: Serum IgE levels were significantly higher in group 4 than in group 3 (P<0.05). IL-4 and IL-5 in BALF were significantly higher in group 4 than in group 3 (P<0.05, respectively). Based on H&E staining, inflammatory cell numbers were significantly greater in group 4 than in the other groups (P<0.05). The number of PAS-positive cells was also significantly greater in groups 3 and 4 than in groups 1 and 2 (P<0.05). The numbers of IL-4 and IL-5-positive cells were higher in group 4 than in group 3 (P<0.05). The number of MUC5AC and TGF-α-positive cells were also higher in group 4 than in group 3 (P<0.05). Conclusions: Our data suggest that ASD increases cytokine expression and mucin production in an allergic murine model. The increased inflammatory reactions were related to cytokine production.

Key Words: Asian sand dust; airway inflammation; mucin

INTRODUCTION

Many studies have demonstrated that air pollution is a major factor in the pathogenesis of respiratory inflammation. The most abundant air pollutants in the atmosphere of urban areas are nitrogen dioxide, ozone, and inhalant particulate matter. It was recently reported that Asian sand dust (ASD), which originates from the Gobi desert in China, influences allergic disease. ASD includes many pollutant materials, especially particulate matter less than 10 μm in diameter (PM10), that can reach small airway bronchioles. ASD particles are generally 0.1-20 μm in diameter and consist of fine particles less than 2.5 μm in diameter and ultrafine particles less than 0.1 μm in diameter. PM10 makes up 53%-71% of ASD, and a high percentage of PM10 is associated with an increased incidence of respiratory disease. The pulmonary function of asthmatic patients deteriorates and their symptoms at night are also somewhat aggravated during the ASD season. Epidemiological studies have demonstrated that ASD events are associated with an increase in daily mortality and hospitalization due to increased respiratory disease. A previous study reported that inflammatory cells are increased in rat bronchoalveolar lavage fluid (BALF) and peripheral blood after exposure to ASD events. ASD enhances the infiltration of eosinophils and lymphocytes into the airway, along with goblet cell proliferation in murine models. ASD may also potentiate common cold symptoms associated with rhinovirus (RV) infection not only by enhancing cytokine secretion, but also by increasing viral replication. However, the pathologic expression of inflammatory cytokines and mucin production in the lungs during allergic inflammation aggravated by ASD remains unknown.

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This study was conducted to investigate the effects of ASD on the expression of inflammatory cytokines and MUC5AC in BALF and lung tissue from an allergic murine model.

MATERIALS AND METHODS

Preparation of particulates

We collected aerosol particles using an air sampler (HV500E, Sibata, Japan) located outside the Gachon University building in Ganghwa near our university on March 16, 2009. Filter packs (Prefilter AP, 124 mm, Millipore, Bedford, MA, USA) were used according to the ASD warning of a PM10 mass concentration of 358.9 μg/m³ and were issued by the Korean Meteorological Agency. The filter paper was removed from the air sampler, torn into pieces, and placed in phosphate buffered saline (PBS) in a 10 mL tube, followed by vortex-mixing. The liquid was then filtered (10 μm pore size). The filtered particulate matter was collected in a 1.5 mL tube after determining its mass. The filtered particulate matter was autoclaved at 121°C for 15 minutes and stored at -20°C until needed. Chemical analysis of ASD particles was performed by the Korea Institute of Ceramic Engineering and Technology.

ASD concentration

The concentrations of each element in ASD were determined using an inductively-coupled plasma optical emission spectrometer (ICP-OES, Optima 5300 DV, Perkin Elmer, Waltham, MA, USA). Endotoxin concentrations were determined using a quantitative chromogenic Limulus Amebocyte Lysate (LAL) test (QCL-1000, Lonza, Walkersville, MD, USA), following the manufacturer’s instructions.

Development of an allergic mouse model and ASD stimulation

All procedures were approved by the Institutional Animal Care and Use Committee of Gachon University of Medicine and Science. Experiments were conducted according to institutional guidelines and federal regulations.

A total of 40 female BALB/c mice (6 weeks of age) were purchased (Central Lab., Animal Inc., Seoul, Korea) and checked for infections. The cages were placed in a conventional room maintained at 20-23°C with 45%-70% humidity. This study adhered to the Gachon University Animal Research Guidelines. The mice in group 1 (G1, n=10) inhaled 1 mL normal saline through a nebulizer (Omron, Japan) for 15 minutes each day during the 7 day study period. Group 2 (G2, n=10) mice inhaled 1 mL of a mixture containing 10 mg ASD and 1 mL normal saline (ASD concentration, 10 mg/mL) using a nebulizer for 15 minutes each day during the 7 day study period. To develop an allergic mouse model, 25 μg OVA (OVA Sigma, St. Louis, MO) and 1 mg Al(OH)₃ gel (Alum, Pierce Chemical Co., Rockford, IL, USA) were mixed in 3 mL normal saline. To perform systemic sensitization, 300 μL of the OVA mixture was injected into the mouse abdominal cavity on the 1st, 7th, and 14th days. The systemically sensitized mice were locally nebulized with 2% OVA for 30 minutes, four times daily starting on the 21st day. Local sensitization was performed in a 40 × 40 × 30 cm acrylic box, which had a side hole connected to the nebulizer, and allergic mice were then divided into group 3 (G3, n=10) and group 4 (G4, n=10). Group 3 mice inhaled 5 mL 2% OVA once every two days (30 minutes each) to sustain the allergic state. Group 4 mice inhaled both 5 mL 2% OVA and 1 mL of a mixture containing ASD and saline (10 mg ASD in 1 mL normal saline) for 30 minutes each day during the 7 day study period (Fig. 1).

Serum and specimens

The mice were sacrificed under intra-abdominal anesthesia with pentobarbital (65 mg/kg) 24 hours after the final stimulation. Blood (300-400 μL) was obtained from the ophthalmic artery by puncturing the orbit. The blood was centrifuged (2,000 rpm, 4°C) to obtain serum after deposition at 4°C for 2-4 hours. BALF was collected by irrigation of the trachea with 2 mL saline using a 26-gauze angiocatheter. Thereafter, 0.5 mL BALF was used for differential cell counts and the remainder was maintained at -20°C for enzyme-linked immunosorbent assay (ELISA). Lung tissue was removed and fixed in 10% formalin. Fixed, paraffin-embedded lung tissues were cut into 4 μm sections and subjected to histological analysis.

OVA-specific IgE in serum

OVA-specific immunoglobulin E (IgE) antibody concentrations were measured using a mouse anti-OVA IgE ELISA kit (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA). Absorbance at

Fig. 1. Allergic mouse model. After intraperitoneal injection of a mixture of 25 μg OVA and 1 mg Al(OH)₃ gel, mice were nebulized with 2% OVA for 4 days. Mice were then stimulated with continuous 2% OVA alone (OVA group) or a mixture of 2% OVA and ASD (OVA+ ASD group). IP, intraperitoneal; ASD, Asian sand dust; OVA, ovalbumin.
450 nm for OVA-specific IgE was measured using a microplate reader (Hitachi U-2000, Tokyo, Japan).

**Total cell and differential cell counts in BALF**
Using a cytopsin, cells were evenly distributed in a 5 mm diameter circle on a slide glass and then stained with Wright-Giemsa. Under 400× magnification, inflammatory cells in five microscopic fields-of-view were counted.

**Measurement of cytokines in BALF**
Cytokine levels in BALF were measured by ELISA using mouse interleukin (IL)-4, IL-5, and IL-13 ELISA kits (Biosource Innl. Inc., Camarillo, CA, USA), and a mouse interferon-gamma (IFN-γ) ELISA kit (PBL BioMedical Laboratory, Piscataway, NJ, USA).

**Hematoxylin–Eosin (H&E) staining**
H&E staining was conducted to evaluate the degree of inflammation. Inflammatory cells were counted at five different sites under 400× magnification.

**Periodic acid–Schiff (PAS) staining**
PAS staining was conducted to evaluate the degree of goblet cell proliferation in the bronchial epithelium. Specimens were cut into 4 μm microsections and placed in periodic acid solution for 5 minutes. After washing with distilled water, the specimens were immersed in Schiff’s fluid for 15 minutes. The specimens were again washed with distilled water for 5 minutes and then placed in hematoxylin fluid for 3 minutes. The number of positive cells was counted at five different sites under 400× magnification.

**Immunohistochemical staining**
Mouse monoclonal antibody, anti-human IL-4 (BioVendor laboratory Medicine, Inc., Czech Republic), purified anti-mouse/human IL-5 Antibody (BioLegend, San Diego, CA, USA), monoclonal anti-human MUC5AC (Neomarker Co., Fremont, CA, USA), and transforming growth factor alpha (TGF-α; Abcam, Cambridge, MA, USA), and transforming growth factor beta (TGF-β; R&D Systems, MN, USA) were used at a dilution of 1:200 at 4°C. Biotinylated anti-rabbit antibody and streptavidin HRP (LSAB kit, DAKO, Glostrup, Denmark) were then added and incubated for 90 minutes each. After color reaction with 3,3-diaminobenzidine tetrahydrochloride (DAB) solution, contrast staining was conducted using hematoxylin. Positive cells were counted and the average of five sites under 400× magnification was calculated. For MUC5AC, the number of positive epithelial cells out of a total of 30 cells was counted.

**Statistical analysis**
Statistical analyses of the pathologic data in the airway and cytokines in BALF were conducted using Fisher’s protected least significant differences (PLSD) test following an analysis of variance (ANOVA; Statview; Abacus Concepts, Inc., Berkeley, USA). Differences between groups were determined to be statistically significant at P<0.05. The correlation coefficients among the numbers of inflammatory cells in BALF and cytokine levels were calculated for each mouse using Fisher’s z-transformation (Abacus Concepts Inc., Piscataway, NJ, USA).

**RESULTS**

**Characteristics of ASD**
The cumulative distribution of 50% of the median sizes was 6.04 μm. The most abundant component was SiO₂ (52.3%). Other major ASD components were Al₂O₃ (16.0%), Fe₂O₃ (5.92%), CaO (4.31%), K₂O (2.51%), MgO (2.48%), and Na₂O (1.68%) (Table 1). Many minor and trace components comprised less than 0.1% of the total. Endotoxin was not detected in autoclaved ASD.

**OVA-specific IgE antibody levels**
OVA-specific IgE levels were significantly higher in group 4 (27.1±1.7 ng/mL) than in group 3 (16.5±0.3 ng/mL) (P<0.05; Fig. 2).

**Total cells and differential counts in BALF**
The total number of cells in BALF was not significantly higher in group 4 than in groups 2 and 3. However, the average number of eosinophils was significantly higher in group 4 (46.7±17.7×10³/mL) than in group 3 (6.3±1.6×10³/mL) (P<0.05; Table 2).

**BALF cytokine concentrations**
IL-4 concentration was significantly higher in group 4 (127.1±4.5 pg/mL) than in group 1 (82.2±5.6 pg/mL) or group 3 (84.6±6.9 pg/mL) (P<0.05 for each). IL-5 concentration was significantly higher in group 4 (39.4±3.8 pg/mL) than in group 1 (32.3±4.2 pg/mL) or group 3 (30.9±4.6 pg/mL) (P<0.05). In addition, IL-13 concentration was significantly higher in group

**Table 1. Analysis of Asian sand dust by the Korea Institute of Ceramic Engineering and Technology**

| Components | Fraction (wt, %) |
|------------|-----------------|
| SiO₂       | 52.3            |
| Al₂O₃      | 16.0            |
| Fe₂O₃      | 5.92            |
| CaO        | 4.31            |
| K₂O        | 2.51            |
| MgO        | 2.48            |
| Na₂O       | 1.68            |
| TiO₂       | 0.73            |
| PO₄        | 0.15            |
| MnO        | 0.12            |
| ZnO        | 0.06            |
| BaO        | 0.05            |
4 (11.5 ± 0.8 pg/mL) than in group 1 (9.3 ± 0.3 pg/mL) (*P < 0.05). However, there was no significant difference in IL-13 concentrations between groups 3 and 4 (*P > 0.05). There were no significant differences in BALF IFN-γ concentrations between the four groups (Table 3).

**H&E staining**

The numbers of inflammatory cells in the bronchi were higher in groups 2 and 3 than in group 1, but the differences were not statistically significant. The average number of inflammatory cells was significantly higher in group 4 (233.4 ± 31.0) than in group 3 (71.4 ± 25.8) (*P < 0.05; Fig. 3). Treatment with OVA alone caused slight eosinophil infiltration, and OVA+ASD caused mild to moderate eosinophil infiltration in the airway (Table 4).

**PAS staining**

The number PAS-positive goblet cells was higher in the bronchial epithelium of groups 3 and 4 after exposure to ASD. The number of positive cells was 28.6 ± 1.3 in group 4 and 10.0 ± 3.1 in group 3. The number of positive cells was significantly higher in group 4 than in the other groups (*P < 0.05; Fig. 4, Table 4).
**Immunohistochemical staining**

The number of IL-4-positive cells was higher in groups 2, 3, and 4 than in group 1. In addition, this number was higher in group 4 (14.2 ± 10.0) than in group 3 (2.4 ± 2.3) \( P < 0.05 \); Fig. 5, Table 4. No IL-5-positive cells were observed in groups 1 and 2, but there were significantly more IL-5-positive cells in group 4 (68.4 ± 20.0) than in group 3 (31.0 ± 14.1) \( P < 0.05 \); Fig. 6. MUC5AC expression was not observed in groups 1 and 2; however,

**Table 4.** Evaluation of pathologic expression of cytokines in the murin airway

| Group         | Eosinophils | IL-4 | IL-5 | TGF-\( \alpha \) | MUC5AC | PAS |
|---------------|-------------|------|------|-----------------|--------|-----|
| Control       | 0           | 0    | 0    | 0               | 0      | 0   |
| ASD           | 0           | 9.6 ± 2.7* | 0    | 0               | 0      | 0   |
| OVA           | 1.3 ± 0.7* | 2.4 ± 2.3* | 31.0 ± 14.1* | 3.6 ± 2.4 | 7.4 ± 5.3* | 10.0 ± 3.1* |
| OVA+ASD       | 4.7 ± 1.2* | 14.2 ± 10.0* | 68.4 ± 20.0* | 17.6 ± 7.4* | 25.8 ± 3.7* | 28.8 ± 1.3* |

\* \( P < 0.05 \) vs. the control group, \* \( P < 0.05 \) vs. the ASD group, \* \( P < 0.05 \) vs. the OVA group.

TGF-\( \alpha \), transforming growth factor alpha; ASD, Asian sand dust; OVA, ovalbumin; HPF, high-power field; PAS, Periodic acid-Schiff.

![Fig. 4.](image1) Periodic acid-Schiff staining. Mucin-containing epithelial cells were significantly increased in the OVA+ASD group as compared with the control and OVA groups (×200). Black arrows indicate PAS-positive cells (original magnification ×200 for A: Control, B: ASD, C: OVA, D: OVA+ASD and ×400 for E: OVA+ASD). \* \( P < 0.05 \) vs. the control group, \* \( P < 0.05 \) vs. the ASD group, \* \( P < 0.05 \) vs. the OVA group.

ASD, Asian sand dust; OVA, ovalbumin, HPF, high-power field.

![Fig. 5.](image2) Immunohistochemical staining of lung tissue for IL-4. IL-4-positive cells (black arrows) were significantly increased in the OVA+ASD group as compared with the control and OVA groups (original magnification ×200 for A: Control, B: ASD, C: OVA, D: OVA+ASD and original magnification ×400 for E: OVA+ASD). \* \( P < 0.05 \) vs. the control group, \* \( P < 0.05 \) vs. the ASD group, \* \( P < 0.05 \) vs. the OVA group.

ASD, Asian sand dust; OVA, ovalbumin; HPF, high-power field.

![Fig. 4.](image3) Periodic acid-Schiff staining. Mucin-containing epithelial cells were significantly increased in the OVA+ASD group as compared with the control and OVA groups (×200). Black arrows indicate PAS-positive cells (original magnification ×200 for A: Control, B: ASD, C: OVA, D: OVA+ASD and ×400 for E: OVA+ASD). \* \( P < 0.05 \) vs. the control group, \* \( P < 0.05 \) vs. the ASD group, \* \( P < 0.05 \) vs. the OVA group.

ASD, Asian sand dust; OVA, ovalbumin, HPF, high-power field.

![Fig. 5.](image4) Immunohistochemical staining of lung tissue for IL-4. IL-4-positive cells (black arrows) were significantly increased in the OVA+ASD group as compared with the control and OVA groups (original magnification ×200 for A: Control, B: ASD, C: OVA, D: OVA+ASD and original magnification ×400 for E: OVA+ASD). \* \( P < 0.05 \) vs. the control group, \* \( P < 0.05 \) vs. the ASD group, \* \( P < 0.05 \) vs. the OVA group.

ASD, Asian sand dust; OVA, ovalbumin; HPF, high-power field.
it was significantly higher in group 3 (7.4±5.3) and group 4 (25.8±3.7) (P<0.05), particularly in group 4 (Fig. 7). No TGF-α-positive cells were observed in groups 1 and 2, but the number of TGF-α-positive cells was significantly higher in group 4 (17.6±7.4) than in group 3 (3.6±2.4) (P<0.05; Fig. 8).

**DISCUSSION**

In this study, ASD increased OVA-specific IgE levels and eosinophil numbers in BALF in an allergic mouse model. The particulate matter in ASD also increased the number of inflammatory cells in the submucosal layer in the OVA+ASD group. This suggests that ASD may increase allergic inflammation by recruiting inflammatory cells. Our result is consistent with a previous study that reported OVA+ASD causes mild to moderate eosinophil infiltration into the airway.13

Our data indicated that IL-4 and IL-5 were significantly expressed not only in BALF, but also in the lung tissue, after ASD stimulation. This suggests that ASD may act as an adjuvant in allergic inflammation by increasing IL-4 and IL-5 release from inflammatory cells. IL-4 and IL-5 are known to increase recruitment of inflammatory cells, especially eosinophils, and airway hyperresponsiveness. IL-13 was significantly increased in group 4, but IFN-γ was not. IL-4 and IL-13 are known to stimulate B lymphocytes to produce IgE antibodies; in contrast, IFN-γ inhibits their production.14 Therefore, it is thought that ASD augments allergic reactions through Th2 cytokines in this allergic model.

Ichinose et al.15 reported that Arizona sand dust and SiO2 synergistically increase levels of eosinophil-relevant cytokines and chemokines such as IL-5 and monocyte chemotactic protein (MCP)-3 in the lungs of murine models. They stated that the aggravated allergic inflammation might be due to mineral components, mainly SiO2. It has recently been demonstrated that titanium dioxide, a representative ultrafine particle, can cause
mucin production. MUC5AC expression is rarely seen in healthy people; however, it is elevated in patients with bronchial asthma and nasal polyposis. IL-4 and IL-9 induce MUC5AC expression. IL-13 increases mucin secretion in the normal airway epithelium and MUC5AC expression in the mouse airway epithelium. Oxidative damage enhances mucin production in airway epithelial cells. Mice exposed to ASD and TiO2 show significant inflammatory changes and oxidative damage to the lungs. DNA damage in peripheral blood mononuclear cells (PBMCs) is also significantly increased in ASD and TiO2-exposed mice. ASD particles cause lung tissue damage through a direct physical effect. In addition, secondarily released cytokines and oxidative stress generated in the lesion may be involved in the development of acute lung toxicity. TGF-α plays a role in the phosphorylation of epidermal growth factor, through which MUC5AC expression is increased in the airway. In this study, TGF-α and IL-13 were increased in the ASD stimulation groups, suggesting that increased TGF-α and cytokines may play a role in MUC5AC expression. Lipopolysaccharide (LPS) and β-glucan absorbed on ASD upregulate expression of pro-inflammatory molecules and thereby play a major role in aggravating lung inflammation. A comparative study has documented that only non-heated ASD increases neutrophils and pro-inflammatory mediators in BALF and that concentrations of toxins and microbes in ASD are not sufficient to cause disease. Our previous study confirmed the absence of LPS in ASD. Therefore, it seems likely that mucus secretion is principally affected by ultrafine PM10 particles rather than microbes.

In this study, we found that ASD increased allergic reactions in the ASD+OVA group. This may be because ASD had a synergistic effect on the production of OVA-induced Th2 cytokines. However, it is unclear whether PM10 itself activates inflammatory reactions. Further studies are needed to confirm our data. In conclusion, the results of this study suggest that ASD may act as an adjuvant in allergic inflammatory reactions and mucin production through increasing pro-inflammatory cytokine levels.

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