Activation of Eosinophils by Rice-Husk Dust Exposure: A Possible Mechanism for the Aggravation of Asthma during Rice Harvest

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KAYABA, H., MEGURO, H., MUTO, H., KAMADA, Y., ADACHI, T., YAMADA, Y., KANDA, A., YAMAGUCHI, K., HAMADA, K., UEKI, S. and CHIHARA, J. Activation of Eosinophils by Rice-Husk Dust Exposure: A Possible Mechanism for the Aggravation of Asthma during Rice Harvest. Tohoku J. Exp. Med., 2004, 204 (1), 27-36 —— Grain dust and other irritants affect the airway of allergic patients in rice-growing area during the harvest. The aim of this study was to elucidate the mechanism of airway hypersensitivity in rice-growing areas during the harvest. Firstly, the effect of rice-husk dust on eosinophil activation was studied. Secondary, the concentration of lipopolysaccharides (LPS), a potent activator of inflammatory cells, in rice-husk dust was measured. Since it is possible for LPS, a component of gram-negative bacterial cell wall, to adhere to the particle of smoke generated from rice-husk dust, LPS contained in the smoke was also measured. Furthermore, chemical irritants contained in the smoke generated from the rice-husk dust were analyzed. Microscopically, the dust contained fine thorns dropped off from the outer sheath of the rice, and irritated the skin, throat and eyes. The grain dust extract increased the expressions of eosinophil activation markers. These up-regulatory effects were largely dependent on LPS. The smoke contained LPS and several chemical irritants such as formaldehyde and acetaldehyde. Rice-husk dust and its smoke, hazardous air pollutants, probably play a major role in the aggravation of airway diseases in agricultural areas. ——— LPS; smoke; formaldehyde; asthma; rice-husk

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Akita Prefecture is located in the northern part of the main island of Japan and is famous for rice. In daily routine clinical work, clinicians working in agricultural areas in Akita are aware of an abrupt increase of patients suffering from asthmatic attacks during the rice-harvesting season. The patients themselves are aware that grain dust and smoke aggravate their airway symptoms. During the harvesting season, which is from September to November, farmers dry the rice crop. In the process of drying the rice, a huge amount of dust is released into the air or lay on the ground inside and outside of the workshop (Fig. 1). It looks like a smooth, fine powder, but it irritates the skin if you handle it. A microscopic view of the dust showed that the main content of the dust is a needle-like structure with a sharp tip coming from the surface of the rice husk. Furthermore, some farmers burn the dust to kill insects and, at the same time, to get ashes to supply minerals to soil. Smoke enshrouds a village during rice-harvesting season. Effects of air pollution on respiratory diseases in industrial areas has been studied in these decades (Sasaki et al. 1998); however, little is known about the effects of rice-husk dust and its smoke as air pollutants in agricultural areas (Muto et al. 1993). To elucidate the mechanism for aggravation of bronchial asthma during rice-harvesting season, we studied the allergic background of patients, components of rice-husk dust, chemical irritants in the smoke, and the biological effect of the rice-husk dust on eosinophils. The role of rice-husk dust and its smoke in asthmatic attacks during the rice-harvesting season were confirmed in this study.

**METHODS**

**Patients**

This study has been approved by the Institutional Committee for Human Studies of Akita University School of Medicine. One hundred patients diagnosed with bronchial asthma were selected...
were analyzed for serum total and specific immune globulin E (IgE) using a radioimmunosorbent test (RIST) and a radioallergosorbent test (RAST), respectively. There were 68 boys and 32 girls aged 10.8±3.6 years on average. The age of onset was 4.5±2.7 years. In 61 patients, a skin scratch test for determining hypersensitivity to rice-husk allergen (Torii Pharmaceutical Co., Ltd., Tokyo) was performed.

Detection of mite allergen

Rice-husk dust was obtained from a farmer who had children suffering from bronchial asthma. Dani Scan (Asahi Beer Pharmaceutical Co., Tokyo), a mite allergen detection kit using immunochromatography, was utilized for the detection of mite allergen. The kit could detect 2 μg of mite allergen contained in 1 g of dust.

Rice-husk dust extract

Twenty ml of phosphate buffered saline (PBS) was added to 20 g of rice-husk dust, and shaken for 4 hours. After centrifugation for 30 minutes, the supernatant was filtered using a 0.45 μm pore-sized filter first, and then a 0.22 μm pore-sized filter.

Smoke extract

Smoke was collected from burning rice-husk dust (3 g) for 15 minutes and was passed through an artificial nose containing an air filter (DAR Hygrobac “S” 352/5877, MALLINCRODT DAR, Mirandola, Italy). The air filter was pulled out of the artificial nose and then dipped in 20 ml of saline and cleaned with an ultrasonic bath (Pasolina DG-1, As One Co., Sendai) for 30 minutes. After centrifugation, the supernatant was used as smoke extract. Room air that passed through a filter for 15 minutes was used to make an extract for control.

Eosinophil donors

A total of 20 habitants in an agricultural area were selected as eosinophil donors, after informed consent was obtained. There were five healthy subjects (four males and one female, aged 26±2.8 years on average) and 15 allergic patients (nine males and six females, aged 25±2.7 years on average). The mean eosinophil count in the peripheral blood of allergic patients and healthy subjects were 383.1 (range 50.0-977.3) and 176.4 (range 59.2-264.0) cells/μl, respectively. The allergic patients were suffering from seasonal mild allergic rhinitis (n=6), atopic dermatitis (n=5) and bronchial asthma (n=4). The blood was obtained at a time when they were asymptomatic and off medication.

Purification of eosinophils

Eosinophils were purified from peripheral blood with a modified cluster of differentiation (CD)-16 depletion method using magnetic beads. After sedimentation of erythrocytes with 6% dextran (McGaw, Irvine, CA, USA), the granulocyte pellet was obtained by density-gradient centrifugation on 1.088 (Pharmacia, Uppsala, Sweden) as modified from the method of Hansel et al. (1991). Mononuclear cells were prepared from the interface. To eliminate mononuclear cells and neutrophils, anti-CD3, anti-CD14, anti-CD19 and anti-CD16 antibody (Nichirei, Tokyo) were added to the cells and incubated for 30 minutes at 4°C. Anti-mouse serum-coated magnetic beads were subsequently added to cells after washing with PBS and incubated at 4°C for 60 minutes. Eosinophils were purified by negative immunomagnetic selection, using magnetically activated cell separator system (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with a high purity (>95%), viability (>99%) and yield.

Cell culture and flowcytometric analysis

Eosinophils were cultured in Roswell park memorial institute medium (RPMI) containing 10% fetal calf serum with or without grain dust extract at a concentration of 1% for 16 hours. Eosinophils were analyzed for their expression of intercellular adhesion molecule-1 (ICAM-1: CD54), very late antigen-4 (VLA-4: CD49d), Mac-1 (CD11b/CD18) and human leucocyte
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antigen (HLA)-DR using a flowcytometer (FACScan, Becton Dickinson & Co., Cockeysville, MD, USA). Eosinophils were suspended in 100 μl of PBS containing 1% of fetal calf serum and 0.1% of NaN3 (Facs buffer) at a concentration of 1×10^5 cells/ml in each tube. One micro grams of monoclonal antibodies against ICAM-1 (84H10, Beckman Coulter Inc., Fullerton CA, USA), VLA-4 (HP2/1, Coulter, FL, USA), Mac-1 (Bear-1, Nichirei, Tokyo) and HLA-DR (G46-6, Becton Dickinson & Co.) were added to each tube and incubated for 30 minutes on ice. Mouse IgG1 (×0931, DAKO, Denmark), and mouse IgG2a (×0943, DAKO) were used as isotype matched controls. After washing, 1 μl of phycoerythrine-conjugated donkey anti-mouse IgG (715-116-151, Jackson Immuno Research Laboratories, Inc., West grove, PA, USA) was added to each tube and incubated for 30 minutes, on ice in a dark. Then, cells were washed with Facs buffer and re-suspended in 500 μl of Facs buffer before flowcytometric analysis. The expressions of these molecules were expressed in delta-mean fluorescence intensity Δ-MFI), the difference in MFI between the sample and the control.

Measurement of lipopolysaccharides (LPS) concentration in rice-husk dust and smoke extract

LPS concentration was assayed with END-SPEC ES-TEST MK (SEIKAGAKU KO-GYO Co., Tokyo) following the manufacturer’s instructions.

Removal of LPS from the extract of rice-husk dust

We dialyzed the rice-husk extract with END-X B15 ENDOTOXIN REMOVAL AFFINITY RESIN (Associates of Cape Cod, Inc., East Falmouth, MA, USA) coated with endotoxin neutralizing protein from limulus polyphemus to remove LPS from the extract following the manufacturer’s instructions.

Measurement of volatile organic compounds in smoke

Smoke generated from 50 g of rice-husk dust was drawn through two kinds of traps continuously by a vacuum pump at a flow rate of each 300 ml/min. Formaldehyde, acetaldehyde and other volatile organic compounds (VOCs) in smoke were trapped with Sep-Pak dinitrophenylhydrazine-silica (Waters Co., Millford, MA, USA) columns for aldehydes and with activated carbon columns for VOCs. A gas chromatography / mass spectrometry / single ion monitor (Shimadzu GC-MS 5050A, Tokyo) was used to determine levels of organic compounds.

Statistical analysis

All measured values were presented as mean ±S.D. Student’s t-test was used for analysis of significant differences between two groups. In comparisons of three or more groups, Fisher’s protected least-significant difference (PLSD) method was used as a post hoc test, regarding p<0.05 as significant after analysis of variance.

RESULTS

Allergic background of the patients

The number of patients with positive RAST scores for mite allergen, animal dust, grass pollen and fungi were 75, 20, 15 and 8, respectively. Hypersensitivity to rice-husk dust was examined using a skin scratch test, because RAST was not available for rice-husk dust allergen. Skin scratch test for rice-husk allergen was negative in 89.9% of the patients and was weakly positive in 11.1% of the patients. The average serum total IgE concentration was 550.7±592.3 (range 41-2700) IU/ml. The serum total IgE levels showed no statistical difference between patients from rice-growing areas and patients from non-agricultural areas (data not shown).

Mite allergens in rice-husk dust

Mite allergen was not detectable in the rice-husk dust. Since most of the patients in rice-growing areas experience aggravation of their re-
Respiratory symptoms during harvesting season, the allergens should be common among the patients if their aggravations were caused by IgE-mediated allergic reactions. This result suggested that mite allergen did not play, at least, a main role in the acute aggravation of respiratory symptoms during rice-harvesting season. There were three possibilities. Firstly, the dust might induce bronchial hypersensitivity via non-allergic pathways. Secondarily, rice-husk dust might contain another allergen common to the patients. And thirdly, the dust might cause nothing but mechanical irritation of the airway.

Effect of the rice-husk dust extract on eosinophils

After 16 hours of incubation under the presence (1% of rice-husk dust extract in the medium) or absence (1% of PBS in the medium) of rice-husk dust extract, eosinophils were analyzed for their expression of surface molecules. In allergic patients, the rice-husk dust extract up-regulated the expressions of Mac-1, ICAM-1 and HLA-DR significantly with p-values of 0.0003, 0.007 and 0.013, respectively. These up-regulatory effects were not seen at 30 minutes, 2 hours and 6 hours of stimulation (data not shown). In healthy sub-

Fig. 2. Effect of rice-husk dust extract on the expression of surface molecules on eosinophils.

In allergic patients (closed circles), eosinophils cultured with rice-husk dust extract for 16 hours showed increased expression of Mac-1(A) (212.4±16.4 ΔMFI), ICAM-1(B) (7.6±1.6 ΔMFI) and HLA-DR(D) (13.1±2.6 ΔMFI) compared to those of the cells cultured without rice-husk dust extract (123.9±7.7, 1.22±0.3 and 8.7±2.0 ΔMFI, respectively). There was no significant difference in the expression of VLA-4 (C) between eosinophils cultured with (41.5±5.0 ΔMFI) or without (42.4±4.4 ΔMFI) rice-husk dust extract. Open squares and error bars represent the mean value and standard error, respectively. In healthy subjects (open circles), rice-husk dust extract up-regulated the expressions of Mac-1(A) (250.2±42.5 ΔMFI) and ICAM-1(B) (11.2±3.3 ΔMFI) but not VLA-4(D) (41.6±5.9 ΔMFI) and HLA-DR(C) (4.6±2.0 ΔMFI) on eosinophils compared to those of the eosinophils cultured without rice-husk dust extract (141.0±5.7, 1.8±0.4, 44.9±3.9 and 5.5±3.3 ΔMFI, respectively).
jects, the rice-husk dust extract up-regulated the expressions of Mac-1 ($p = 0.049$) and ICAM-1 ($p = 0.048$) but not HLA-DR. There was no significant change in VLA-4 expression on the eosinophils obtained from either allergic patients or healthy subjects (Fig. 2). These results indicated the presence of a potent eosinophil activator in rice-husk dust. IgE seemed not to be a main factor, because in a study in vitro, we confirmed that IgE or FcεRI cross-linking on eosinophils caused no change on the surface expressions of these molecules (data not shown).

**LPS in the rice-husk dust and its smoke**

Since LPS was a potent inflammatory cell activator, we measured the concentration of LPS in rice-husk dust extract. High concentrations of LPS were detected from extracts of rice-husk dust ($135.0 \times 10^3$ to $621.1 \times 10^3$ pg/ml) and its smoke ($112.6$ to $361.9$ pg/ml). LPS concentration in PBS was below the lower limit ($3.9$ pg/ml) of the detection test. Compared with extracts from rice-husk dust or its smoke, quite low concentration of LPS ($<3.9$ pg/ml) was detected from that of room air.

**Effect of LPS-adsorbed rice-husk dust extract on the expressions of Mac-1 and ICAM-1 on eosinophils**

To take the next step forward, we dialedyzed the rice-husk extract with END-X B15 ENDOTOXIN REMOVAL AFFINITY RESIN coated with endotoxin neutralizing protein from *limulus polyphemus*, and removed to 99.9% of LPS from the extract. The up-regulations of Mac-1, ICAM-1 and HLA-DR expressions on eosinophils from allergic patients were significantly diminished after adsorption of LPS from the rice-husk dust extract (Fig. 3).

**Volatile organic compounds in smoke**

The up-regulation of Mac-1, ICAM-1 and HLA-DR on eosinophils was not seen until 16 hours after incubation with rice-husk dust extract. However, patients often experienced asthmatic attacks induced soon after they inhaled the smoke generated from burning rice-husk dust. We analyzed organic compounds such as formaldehyde, acetaldehyde and phenols. These compounds are known to induce airway hypersensitivity (Delfino 2002; Rumchev et al. 2002; Sadakane et al. 2002). The smoke generated from 50 g of rice-husk dust contained more than $1.4$ mg/m$^3$ of formaldehyde and $137$ μg/m$^3$ of acetaldehyde (Fig. 4). Furthermore, several kinds of hazardous organic compounds such as phenols were also detected with concentrations ranging from $10 \sim 10^3$ mg/m$^3$.

**DISCUSSION**

Air pollution in industrialized area is a concern of wide variety of people; however, air pollution in restricted areas such as rice-growing areas and volcanic areas (Kariya et al. 1992) attracted little attention and little is known among general population.

Aggravation of airway symptoms has been experienced among people living in rice-growing areas in every rice-harvesting season. In 1984, Lim et al. (1984) from Malaysia reported a clinical condition caused by rice-husk dust, referring to it as “Rice Millers’ Syndrome”. The manifestation includes acute and chronic irritant effects affecting eyes, skin, and respiratory tract. An increased prevalence rate for bronchial asthma and chronic bronchitis is reported in rice farmers exposed to rice-husk dust (McCurdy et al. 1996), but the biological mechanism of rice-husk dust-induced asthma is not well understood. IgE-mediated allergic reaction (Ingram et al. 1979), LPS-induced airway sensitivity (Oliver et al. 1989; Kline et al. 1999) and mechanical irritation of the airway may be included in the mechanism. However, IgE-mediated allergic reaction was not, at least in our series, a main pathway, because there were no detectable mite-allergens in rice-husk dust and the percentage of the patients positive for rice-husk dust extract was only 11%.

Rice-husk dust extract was proved to up-regulate the expressions of Mac-1, ICAM-1 and HLA-DR on eosinophils in the present study.
Furthermore, LPS was revealed to be a main factor responsible for these effects, because LPS-adsorbed rice-husk dust extract showed a marked reduction of up-regulatory effect on the expression of surface molecules on eosinophils. Mac-1 expressed on eosinophils plays important roles in eosinophil function in migration (Larangeira et al. 2000) and degranulation (Kato et al. 1998). Up-regulation of HLA-DR on eosinophils obtained from allergic patients may enhance eosinophil-lymphocyte interaction in immune response (Weller et al. 1993).

LPS acts as a strong activator for cells participating in inflammatory reaction. LPS induces a release of tumor necrosis factor (TNF)-α and eosinophil cationic protein (ECP) from eosinophils in a CD14 and toll-like receptor (TLR)4-dependent fashion (Plötz et al. 2001). Czech et

Fig. 3. Effect of LPS adsorption from rice-husk dust extract.
Rice-husk dust extract lost its up-regulatory effect on the expression of ICAM-1, Mac-1 and HLA-DR after LPS adsorption. Data is expressed in percentage of the ΔMFI of the samples cultured with rice-husk dust extract or LPS-adsorbed rice-husk dust extract to that of the samples cultured without rice-husk dust extract. Error bars represent standard error. In the expressions of Mac-1, ICAM-1 and HLA-DR, the percentage went from 181.0, 454.2 and 153.6 on average, respectively, in rice-husk dust treated eosinophils to 118.8, 166.7 and 99.5 on average, respectively, when LPS was adsorbed from rice-husk dust extract.
Eosinophils obtained from allergic patients were used in this series.
A: Mac-1, B: VLA-4, C: ICAM-1, D: HLA-DR.
al. (1993) reported TNF-α-induced up-regulation of ICAM-1 on eosinophils. TNF-α may act in an autocrine or paracrine fashion on eosinophils after stimulation with LPS. In vivo, LPS induces secretion of cytokines and chemokines from the cells participating in inflammatory reaction including eosinophils. Mast cells produce TNF-α, IL-1β, IL-6 and IL-13 upon stimulation of TLR4, a ligand of LPS (Supajutura et al. 2002). Nasal fibroblasts produce RANTES (regulated upon activation, normal T expressed and presumably secreted) and granulocyte/macrophage colony-stimulating factor (GM-CSF) by LPS stimulation (Nonaka et al. 1999). LPS is reported to induce eosinophil accumulation (Peden et al. 1999, Penido et al. 2001) and prolong eosinophil survival (Meerschaert et al. 2000). Furthermore, grain dust inhalation induces IL-8 production by bronchial epithelial cells, and increases polymorphonuclear cell accumulation in the airway (Becker et al. 1999; Park et al. 1999). Thus, LPS contained in rice-husk dust acts on cells orchestrating airway inflammation. LPS possibly plays important roles inducing airway inflammation and asthmatic attacks during the rice-harvesting season in agricultural areas. Moreover, it is reported that grain dust directly acts on mast cells and basophils to release significant amount of histamine (Alam et al. 1988).

Air pollution by the smoke generated from rice-husks spreads into neighboring area. In the present study, not only LPS, but also chemical irritants such as formaldehyde and acetaldehyde were shown to generate from burning rice-husk dust at high concentrations. Formaldehyde, a suspected chemical irritant responsible for sick building syndrome, is reported to enhance eosinophilic inflammation (Sadakane et al. 2002) and increase the adhesiveness between endothelial cells and eosinophils (Kim et al. 2002).

Therefore, the present study suggested that both rice-husk dust and its smoke have ill effects for the asthmatic patients living in rice growing area. Thus, the presence of mechanical irritant, biological activator for inflammatory cells and chemical airway irritant in rice-husk and its smoke was shown in the present study. More attention should be paid to rice-husk dust and its

Fig. 4. Gas chromatographic and mass spectrometric analysis of formaldehyde and acetaldehyde dinitrophenylhydrazones in the smoke generated from rice-husks. A part of gas chromatograph obtained from the smoke generated from rice-husk dust was shown. High amount of formaldehyde (FA-DNPHz) and acetardehyde (AA-DNPHzs) were detected.
smoke as hazardous air pollutants in rice-growing areas.

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References


