Inhibitory Effect of DA-9201, an Extract of *Oryza sativa* L., on Airway Inflammation and Bronchial Hyperresponsiveness in Mouse Asthma Model

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Asthma is one of the major public health problems worldwide and the morbidity and mortality of asthma has increased in the past two decades. Accumulating data suggest that unnecessary immune responses and inflammation should be suppressed to treat asthma. The purpose of this study is to investigate the anti-asthmatic effects of DA-9201, an ethanolic extract of black rice (*Oryza sativa* L. var *japanica*), on an ovalbumin (OVA)-induced mouse model of asthma. Balb/c mice immunized with OVA were administered with DA-9201 (30, 100 or 300 mg/kg, p.o.) or dexamethasone (3 mg/kg, p.o.) and challenged with 1% aerosolized OVA for 30 min. The effects on airway inflammation, airway hyperresponsiveness (AHR), antibody profiles and cytokines were evaluated. DA-9201 treatment significantly reduced the number of eosinophils in bronchoalveolar lavage fluid (BALF) and ameliorated the AHR. Lung histological features also showed that DA-9201 reduced airway inflammation. Furthermore, DA-9201 treatment decreased IFN-γ as well as IL-4, IL-5 and IL-13 levels in the supernatant of cultured splenocytes, and suppressed the level of OVA-specific IgG, IgG2a, IgG1 and total IgE in plasma. DA-9201 showed anti-asthmatic effects by suppressing unnecessary immune responses, airway inflammation, eosinophilia, AHR and IgE level. These results suggest DA-9201 might be beneficial for the treatment of asthma.

Key words *Oryza sativa* L.; DA-9201; airway inflammation; airway hyperresponsiveness; asthma

**MATERIALS AND METHODS**

**Mice** Female Balb/c mice (6 weeks) were obtained from Charles River Japan. They were housed under a 12-h light/dark cycle (lighting: 07:00 to 19:00), in the temperature range of 23 ± 2 °C, at a relative humidity of 60 ± 10%, with ventilation (turnover frequency of 15—20 times per hour) and a light intensity of 150—600 Lux. All experiments were performed in accordance with the institutional Standard Operation Procedure for Animal Care and Experiments (SOP-ANC) of the Dong-A Pharmaceutical Company and with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health.

**Reagents** Acetyl-β-methylcholine chloride (methacholine, Mch) and dexamethasone-water soluble (DEXA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). OVA (chicken egg albumin grade V) and aluminum hydroxide gel (alum) were also purchased from Sigma-Aldrich. All other chemicals or reagents were commercial and of the highest quality available.

**The Preparation of DA-9201** The black rice (*Oryza sativa* L. var. *japonica*) was purchased from the National Agricultural Cooperative Federation (Geochang, S. Korea). The black rice (8.00 kg) was processed by using a household rice-polishing machine (Bio SangSang, Korea) to obtain the rice bran (1.348 kg). Then, the rice bran was extracted with 70% ethanol (6.74 l) for 24 h at room temperature with agitation. The extract was filtered through filter paper (Advantec, Japan) and the filtrate (5.13 l) was concentrated at 60 °C under vacuum using an evaporative system and lyophilized at

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−40 °C to dryness; 77.80 g (yield: 5.77%) of extract powder was obtained. The dried extract, DA-9201, was suspended in a 1% hydroxyprophylmethylcellulose (HPMC) solution for oral administration.

The HPLC Analysis of DA-9201 The dried ethanol extract, 10 mg of DA-9201, was dissolved in 1 ml of mobile phase followed by filtration using a 0.45 μm syringe filter. Standards of several anthocyanins including cyanidine-3-O-β-glucopyranoside, peonidin-3-O-β-glucopyranoside, delphinidin-3-O-β-glucopyranoside and pelargonidin-3-O-β-glucopyranoside were obtained from Polyphenols Laboratories (Hanabryggene Technology Centre, Norway). Standards of phenolic acids such as protocatechuic acid and ferulic acid were obtained from Sigma-Aldrich.

The chromatographic systems consisted of Dionex (Sunnyvale, CA, U.S.A.) equipped with a P580 pump, ASI-100 automated sample injector and UV detector. Separations were performed using 5 μm Inertsil ODS column (250×4.6 mm ID; GL Science Inc., Japan) with 1 mM phosphoric acid/methanol (85 : 15, v/v) for protocatechuic acid, and 0.5% trifluoroacetic acid/acetone (85 : 15, v/v) was used for anthocyanins at a flow rate of 1 ml/min. Wavelengths used for the identification of protocatechuic acid and anthocyanins were 254 nm and 520 nm, respectively. To quantify the ferulic acid, the HPLC-MS analyses were carried out using a Waters Alliance 2975 LC system (Waters Co., U.S.A.) coupled to a mass spectrometer. DA-9201 was separated on an Atlantis C18 column (5 μm, 2.1 mm i.d.×100 mm, Waters Co., U.S.A.) using an isocratic elution of acetone/triethylamine (50 : 50, v/v) at a flow rate of 0.2 ml/min. The temperatures for the column and autosampler tray were 40 °C and 15 °C, respectively. The eluent was introduced directly into the negative ionization electrospray source of a tandem quadrupole mass spectrometer (Quattro Micro, Micromass UK Ltd., U.K.). The ion source and desolvation temperature were held at 120 °C and 40 °C, respectively. The optimum cone voltages and collision energy were 20 eV and 18 V, respectively. Selected reactions monitoring (SRM) using the precursor product ion transition energy were 20 eV and 18 V, respectively. The optimum cone voltages and collision energy were 20 eV and 18 V, respectively. The ion source and desolvation temperature were held at 120 °C and 40 °C, respectively. The optimum cone voltages and collision energy were 20 eV and 18 V, respectively.

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Experimental Design Airway eosinophilia was elicited by a modified protocol (Fig. 1). Mice were immunized intraperitonially with 50 μg OVA mixed with 1 mg alum as an adjuvant in a total volume of 0.1 ml per mouse. Sensitized mice were immunized again 10 d after the primary immunization. Normal control mice were injected intraperitoneally with phosphate buffer saline (PBS, pH 7.0). DA-9201 was administered orally from day 1 to day 23 and from day 10 to day 23, respectively.

Bronchoalveolar Lavage Under the ether anesthesia mice were bled and sacrificed. BALF was collected by lavaging the trachea by three times with total 1.2 ml of pyrogen-free PBS containing 0.05 mM EDTA. The lavage fluid was centrifuged at 400 g for 3 min at 4 °C, and the cells were separated from the fluid. The fluid was stored at −20 °C until it was analyzed. The cells were re-suspended in PBS containing 0.05 mM EDTA, and the total number of viable cells was determined using a hemacytometer. Differential cell numbers were determined with cytocentrifugation preparations, followed by Giemsa staining.

Preparation of Splenocytes Single cell suspensions were prepared from spleens placed in RPMI 1640 medium and erythrocytes were lysed with RBC lysis buffer (NH₄Cl 0.16 M 90%, Tris 0.17 M 10%, pH 7.2). After washing, the cells were re-suspended in RPMI 1640 containing 10% FBS and 100 U/ml of penicillin–streptomycin. Two-hundred microliters of splenocytes suspension was plated at 10⁶ cell per well with 50 μg/ml of OVA in 96-well round-bottom microtiter plates. The final volume of each well Cells were cultured at 37 °C with 5% CO₂ for 60 h.

Enzyme-Linked Immunosorbent Assay for Cytokines and Total-IgE IL-4, IL-5, IL-13 or IFN-γ in BALF and in the supernatant of cultured splenocytes were determined by enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (R&D Systems, MN, U.S.A.). Total IgE in plasma, BALF and the supernatant of cultured splenocyte from each animal were also analyzed according to the manufacturer’s instructions (SHIBAYAGI Co. Ltd., Japan).

ELISA for Ag-Specific IgG, IgG2a and IgG1 in Plasma
RESULTS

DA-9201  DA-9201, ethanolic extracts of black rice bran, was black to purple colored-powder. The amount of the standard materials contained in DA-9201 is shown in Table 1. Cyanidine-3-O-β-glucopyranoside, peonidine-3-O-β-glucopyranoside, pelargonidine-3-O-β-glucopyranoside, protocatechuic acid and ferulic acid were contained 1.49%, 0.30%, 0.47%, 0.30% and 0.24%, respectively.

Effect of DA-9201 on the Total and Differential Cell Numbers in BALF  The number of total cells in BALF was significantly increased in OVA control mice (Fig. 2A). However, the cell number was reduced in a dose-dependent manner by the administration of DA-9201. Furthermore, while the proportion of eosinophils in OVA control was significantly increased (Fig. 2B), treatment with DA-9201 induced a dose-dependent decrease in the ratio of eosinophil. The administration of DEXA at the dose of 3 mg/kg also significantly reduced the total and differential number of eosinophils in BALF.

Effects of DA-9201 on Pathological Changes in Lung  The scores of total lung inflammation including airway edema and cellular infiltration were significantly increased in OVA control mice when compared with the normal control mice (Fig. 3). These responses were ameliorated in a dose-dependent manner by the administration of DA-9201. Inflammatory score was significantly decreased at the dose level of 100 mg/kg.

Table 1. The Amounts of Standard Materials in DA-9201

<table>
<thead>
<tr>
<th>Standard materials</th>
<th>Quantity (%)</th>
<th>Retention time</th>
</tr>
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<tbody>
<tr>
<td>Cyanidine-3-O-β-glucopyranoside</td>
<td>1.49</td>
<td>5.63—5.65</td>
</tr>
<tr>
<td>Peonidine-3-O-β-glucopyranoside</td>
<td>0.30</td>
<td>9.15—9.53</td>
</tr>
<tr>
<td>Delphinidine-3-O-β-glucopyranoside</td>
<td>0.00</td>
<td>3.95—4.05</td>
</tr>
<tr>
<td>Pelargonidine-3-O-β-glucopyranoside</td>
<td>0.30</td>
<td>8.15—8.45</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>0.47</td>
<td>2.8</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.24</td>
<td>6.1</td>
</tr>
</tbody>
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Lung Histology  Histopathological examination was performed on lungs that were removed and fixed with 10% phosphate-buffered formalin. The tissues were embedded in paraffin, sectioned at a thickness of 5 µm, stained with hematoxylin and eosin (H&E), and analyzed through use of light microscope. The inflammatory score was obtained from the following criteria; inflammation including airway edema and cellular infiltration was graded as none (score 0), mild (score 0.5), moderate (score 1), or severe inflammation (score 1.5).

Statistical Analysis  All statistical analyses were performed using SigmaStat® for Windows 2.0 software (Jandel Corporation, U.S.A.). The ANOVA test was used for the comparisons between the experimental groups and within each test group. All the data is expressed as a mean±standard error of the mean (S.E.M.). The comparison between the group means was accomplished using a Dunnett’s multiple range test at a significance level of p=0.05.
DA-9201 300 mg/kg, which was comparable to that of the DEXA-treated group.

**Effects of DA-9201 on AHR** In OVA control mice, the percent Penh produced by the exposure to methacholine was significantly increased when compared with that of the normal control mice. DA-9201 treatment showed a significant and dose-dependent decrease in AHR. While the dose of level of DA-9201 30 mg/kg or 100 mg/kg did not show the statistical significance (data not shown), 300 mg/kg of DA-9201 significantly reduced the AHR, which was comparable to that of the mice treated with DEXA (Fig. 4).

**Effects of DA-9201 on Levels of IL-4, IL-5, IL-13 and IgE in BALF** Enzyme immunoassay revealed that levels of IL-4, IL-5, IL-13 and IgE in BALF were significantly increased at 24 h after the last OVA inhalation in the OVA control mice (Figs. 5A—D). The levels of these cytokines and IgE were decreased by the treatment with DA-9201.

**Effects of DA-9201 on Levels of IL-4, IL-5, IFN-γ and IgE in the Supernatant of Cultured Splenocytes** While the levels of IL-4 and IL-5 were significantly increased in OVA control, the levels of IL-4 and IL-5 in DA-9201-treated groups were significantly decreased in a dose-dependent manner (Figs. 6A, B). The level of IFN-γ was not significantly decreased by the administration of DA-9201 when compared with OVA control (Fig. 6C). Furthermore, DA-9201 treatment significantly and dose-dependently decreased the IgE level (Fig. 6D).

**Effects of DA-9201 on Antibody Responses in Plasma** As shown in Fig. 7, DA-9201 at the dose of 300 mg/kg showed a significant suppression in levels of plasma OVA-specific IgG, IgG2a, IgG1 and total IgE.

**DISCUSSION**

Accumulating data have shown that unbalanced and aberrant Th2 inflammation and subsequent eosinophil infiltration into lung are the main cause of allergic asthma. In fact, IL-4, one of the representative Th2 cytokines, has been shown to promote B cell switching to IgE production with IL-13, which enhance mucus production and AHR. IL-5, another Th2 cytokine, is also the primary determinant of eosinophil priming, activation, recruitment, growth, differentiation and survival. Moreover, infiltrated eosinophils have dense intracellular granules that are sources of inflammatory proteins. Major basic protein, one of those proteins, can directly damage airway epithelium, intensify bronchial responsiveness, and cause degranulation of basophils and mast cells. Therefore, it appears evident that the inflammation caused by Th2 cells and eosinophils and subsequent responses initiate and maintain asthma. From this point of
view, anti-inflammatory therapies have become important therapeutic strategies for asthma treatment. Corticosteroids are the most potent, non-specific anti-inflammatory agents and they are known to produce substantial improvement in lung functions of asthmatic subjects. However, frequently reported side effects make the compliance poor. Furthermore, taking it into consideration that asthmatics should have long-term anti-inflammatory therapies, there is a need for safe and potent drug which has fewer side effects.\(^5\) In this study, we investigated possible therapeutic effects of DA-9201, an extract of black rice (\textit{Oryza sativa L. var. japonica}), on allergic asthma using a mouse model. We found that the treatment of DA-9201 exhibited anti-inflammatory properties as evidenced by the reduction of eosinophil infiltration and Th2 cytokines. Lung histological examination also showed that DA-9201 reduced airway inflammation. Furthermore, it is ex-

Fig. 6. Effects of DA-9201 on Levels of IL-4, IL-5, IFN-\(\gamma\) and IgE in the Supernatant of Cultured Splenocytes

The levels of IL-4 (A), IL-5 (B), IFN-\(\gamma\) (C) and IgE (D) in the supernatant of cultured splenocytes were quantified by ELISA after culture with OVA for 60 h. Bars represent mean \(\pm\) S.E.M. (\(n=9\)). \(^a p<0.05\) vs. normal control, \(^b p<0.05\) vs. OVA control. SAL: negative control, OVA: positive control, 30; DA-9201 30 mg/kg, 100; DA-9201 100 mg/kg, 300; DA-9201 300 mg/kg, DEXA; dexamethasone 3 mg/kg.

Fig. 7. Effects of DA-9201 on OVA-Specific IgG, IgG2a, IgG1 and Total IgE Levels in Plasma

Plasma was obtained at 24 h after the last OVA challenge. OVA-specific IgG (A), IgG2a (B), IgG1 (C) and total IgE (D) were determined by ELISA. Data represent mean \(\pm\) S.E.M. (\(n=9\)). \(^a p<0.05\) vs. normal control, \(^b p<0.05\) vs. OVA control. SAL; negative control, OVA; positive control, 30; DA-9201 30 mg/kg, 100; DA-9201 100 mg/kg, 300; DA-9201 300 mg/kg, DEXA; dexamethasone 3 mg/kg.
ected that DA-9201 is particularly safe without side effects because DA-9201 induced no toxicological changes in several toxicological studies (data known shown), which was comparable to previous study showed that administration with black rice extract to rats induced no physiological differences up to 10 g/kg. Thus, DA-9201 has advantages over other anti-inflammatory agents in that it is particularly safe and an effective oral treatment.

The idea that allergic inflammation in asthma arises from an imbalance between Th1 and Th2 cells has focused attention on the representative Th1 cytokine, IFN-γ. Since IFN-γ has been reported to inhibit the synthesis of IgE and the differentiation of precursor cells to Th2 cells, increased level of IFN-γ has been used to explain the anti-asthmatic effects of therapeutic agents. However, other data have suggested that IFN-γ contribute to the activation of eosinophils and thus be likely to augment inflammation. Thus it seems to be reasonable that unnecessary immune response caused by Th1 cells as well as inflammation mediated by Th2 cells and eosinophils should be suppressed to treat asthma. We revealed that DA-9201 down-regulated Th2 cytokine production both in cultured splenocytes and BALF in a dose-dependent manner. Especially, at the dose of 300 mg/kg, IL-4 and IL-5 production in cultured splenocytes and IL-4 in BALF was significantly decreased although IL-5 and IL-13 levels in BALF did not show statistical significance. Likewise, DA-9201 suppressed OVA-specific IgG1 produced by help of Th2 cells in a dose-dependent manner with the statistical significance at dose level of DA-9201 300 mg/kg. Furthermore, DA-9201 suppressed significantly OVA-specific IgG and IgG2a production, which was produced by help of Th1 cells. However, DA-9201 did not directly and significantly affect IFN-γ production in cultured splenocytes when compared with OVA control. Taking these results into consideration, immune responses mediated by Th2 cells and B cells were markedly suppressed by the administration of DA-9201. However, the effect of DA-9201 on immune response mediated by Th1 cells might be minimum. Further studies are needed to elucidate the exact mode of action of DA-9201.

Unlike other isotypes, IgE was formed by allergen binds to Fc(epsilon)RI on mast cells and basophils without allergen, and the cross-linking of mast cells or basophils-bound IgE by allergen induces the activation of membrane and cytosolic pathways that cause the release of preformed mediators such as histamine and initiates the synthesis of arachidonic acid metabolites. A recombinant humanized monoclonal antibody against IgE, omalizumab, has been used as an anti-asthmatic treatment. DA-9201 also suppressed IgE levels in plasma, BALF and the supernatant of cultured splenocytes. Therefore, it is expected that DA-9201 can alleviate IgE-mediated subsequent responses as well as airway inflammation and eosinophilia.

Although the pharmacologically active components of DA-9201 have not yet been characterized and its chemical composition is also still not completely elucidated, DA-9201 suppressed airway inflammation and IgE level in this experimental model of asthma. This effect was ultimately accompanied by the reduction of airway hyperresponsiveness. Based on these results, we concluded that DA-9201 might be beneficial for the treatment of asthma.

REFERENCES