



Oral administration of allergen extracts from *Dermatophagoides farinae* desensitizes specific allergen-induced inflammation and airway hyperresponsiveness in rats

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ABSTRACT

Clinically sublingual immunotherapy (SLIT) by using allergen extracts effectively alleviates the symptoms of allergic rhinitis and asthma. Supposed that oral administration of high-dose of allergen extracts imitates SLIT and may prevent IgE-related responses in allergic diseases, we investigated the effects of oral administration of allergen extracts from *Dermatophagoides farinae* (Derf) on allergen-induced inflammation and airway hyperresponsiveness (AHR) in a model of asthmatic rat. After administration to the specific Derf-sensitized rats with Derfdrop solution containing Derf1 and Derf2 extracts derived from Derf, the effects of Derfdrop on AHR, inflammatory cell accumulation, cytokine production in the bronchoalveolar lavage fluid and lung tissue, as well as serum IgE and IgG levels were investigated. Results indicated that Derfdrop not only dose-dependently prevented the AHR in response to methacholine, but also significantly reduced the serum total and allergen-specific IgE levels, all the maximal effects were achieved at dose of 5 mg/kg/d, and were as comparable as those of dexamethasone at dose of 1.0 mg/kg/d. Furthermore, oral administration of Derfdrop not only dose-dependently elevated allergen-specific serum IgG levels and reduced total and allergen-specific IgE levels, but also normalized the imbalance between the Th1 cytokine, IFN- γ and Th2 cytokine, IL-4. Finally, oral administration of Derfdrop significantly reduced Goblet cell hyperplasia and eosinophilia in the Derf-sensitized allergic rat model. These data suggest that Derfdrop effectively improves specific allergen-induced inflammation and AHR in Derf-sensitized and -challenged rats and provide with the rationale for clinical SLIT by using Derfdrop in a specific allergen-induced asthma.

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1. Introduction

Asthma is one of the most common inflammatory lung diseases, its prevalence and incidence have increased in many developed and developing countries [1]. Inhalation of allergens evokes deleterious immune and inflammatory responses in the airway, which further leads to airway hyperresponsiveness and airway remodeling [1]. A known risk factor for the development of atopic asthma is exposure and sensitization to the house dust mites. House dust mites are important sources of indoor allergens associated with asthma and other allergic disorders [2]. The most common and important aeroallergens among dust mite species are *Dermatophagoides farinae* (Derf) and *Dermatophagoides pteronyssinus* (Derp). Up to date, several clinically important allergens such as Derp1, Derp2, Derf1 and Derf2 have been identified in crude mite extracts [3]. Recombinant Derp1, Derp2, Derf1 and Derf2 have been shown to exhibit allergic activity in humans and animals comparable with native products prepared from crude mite extracts [4–7]. Since sensitivity to these allergens is present in 60% to 80% of patients with dust mite allergies, Derf and

Derp could be valuable tools for the diagnosis and immunotherapy of allergic asthma and rhinitis caused by house dust mites.

Unlike symptomatic treatment, allergen-specific immunotherapy not only improves symptoms, but also modifies the natural course of the disease in patients with allergic rhinitis and mild asthma [8,9]. Since traditional therapies, such as subcutaneous immunotherapy (SCIT), have the risk of serious adverse reactions (e.g., anaphylaxis, death), there has been developing more convenient alternatives to subcutaneous injections in order to improve safety and compliance, sublingual immunotherapy (SLIT) was developed for these purposes [10]. Many clinical studies have demonstrated that SLIT is a safe and clinically effective treatment in patients sensitive to house dusts with rhinitis and asthma [8,10–14]. However, the detailed effects and efficacy of Derf extracts by SLIT on modulation of immunoresponse in asthma are not fully understood, especially, how SLIT affects IgG and IgE levels in specific allergen-induced asthma and whether SLIT works by modulating the Th1- or Th2-biased allergic immune response deserve further study [15]. Animal models are valuable supplement to clinical and in vitro studies, when we explain the mechanism of allergic asthma, and these animal models might provide the clues concerning the effects and mechanisms of current treatment of specific allergen-induced asthma, which is greatly vital for generation of new and more effective treatment for the

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disease [15]. In the present study, we used Derfdrop solution containing Derf1 and Derf2 extracts derived from *D. farinae* as an example of allergen extracts from house dust mite, and orally administrated the Derf-sensitized and -challenged rats with Derfdrop solution to imitate the clinical SLIT for asthma. The effects of Derfdrop on airway hyperresponsiveness (AHR), cytokine production, inflammatory cell accumulation in the bronchoalveolar lavage fluid (BALF) and lung tissue, serum total and allergen-specific IgE and IgG levels were investigated. Our data suggest that oral administration of Derfdrop effectively improves specific allergen-induced inflammation and AHR in Derf-sensitized rats, and provide the rationale for clinical SLIT by using allergen extracts for treatment of asthma.

2. Materials and methods

2.1. Animals

Six-week-old female Sprague–Dawley rats were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Certificate No.: SCXK2003-0003, Shanghai, China). All animals were housed in a room maintained at $23 \pm 2^\circ\text{C}$ with $50 \pm 10\%$ humidity and a 12-h light 12-h dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). Animals were allowed free access to tap water and regular rodent chow. Rodent chow was withheld 8 h before the experiments. All the animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. Animals were terminally euthanized by inhalation of CO_2 .

2.2. Antigen

Allergens derived from *D. farinae* (Derf) were purified from whole ground mites by defatted, extracted in 0.125 M ammonium bicarbonate, and further dialyzed with distilled water. Additional purification was performed by using DEAE ion exchange chromatography and gel filtration, and the preparation (Derfdrop solution) containing $>55\%$ of group 1 allergen (Derf1) and $>20\%$ of group 2 allergen (Derf2) (Zhejiang Wowu Biotech Co. Ltd., Hangzhou City, China) was used for oral administration, however, the preparation containing $>70\%$ of Derf1 and $>55\%$ of Derf2 was used for sensitization.

2.3. Experimental procedure

Ninety-six rats were used in the experiments, and sensitization was performed as described previously [16]. Briefly, $10\ \mu\text{g}$ of Derf allergen (containing $>70\%$ Derf1 and $>55\%$ Derf2) emulsified in 0.5 ml of 4% aluminum hydroxide adjuvant was subcutaneously injected in the footpad, neck, back, and groin of each animal, and the injection was performed on days 1, 10 and 20, respectively. Negative control rats (control group) were subcutaneously injected with 0.5 ml of 4% aluminum hydroxide adjuvant only. From day 28, control and sensitized rats were placed in a plastic box and challenged for 30 min daily via the airways with aerosolized Derf allergen (1% in saline) or equal volumes of saline (control group) by a jet nebulizer (BARI Co. Ltd., Germany) for 7 consecutive days (Fig. 1). AHR to methacholine (Mch, Sigma, St. Louis, MO) was measured 24 h after the last challenge in five groups of rats ($n=10$ in each group). In another five groups of rats ($n=8-10$), sera were harvested for assessment of immunoglobulin levels, BALF was prepared from the right lung, and was used for inflammatory cell counting and cytokine measurement. The paraffin section from the left upper lobe was used for histological assessment, and the left lower lobe was homogenized for cytokine measurements.

2.4. Oral administration of Derfdrop

Since it is hard to perform SLIT in the animals, we imitated clinical SLIT by feeding Derf solution into animal mouth by a syringe until the

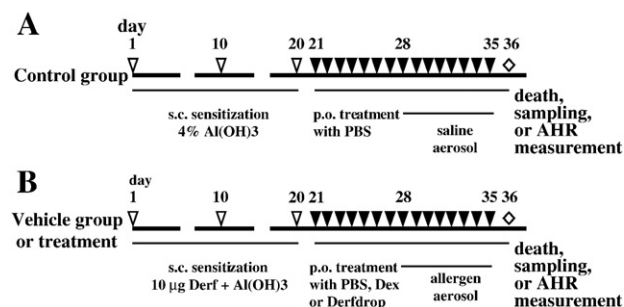


Fig. 1. Experimental procedure. Rats were subcutaneously (s.c.) sensitized for three times with $10\ \mu\text{g}$ of Derf allergen emulsified in 0.5 ml of 4% aluminum hydroxide adjuvant. Negative control rats (Control group) were injected with 0.5 ml of 4% aluminum hydroxide adjuvant alone following the same protocol. 28 days later, all animals were placed in a plastic box and challenged with antigen via the airways with aerosolized Derf allergen (1% in saline) or equal volume of saline (Control group) in a jet nebulizer for 30 min, daily, for 7 consecutive days. Oral treatment was started at 24 h after the last immunization. The 0.5 mg/kg/d Derfdrop group, each rat (wt. 200 g) was fed with 0.2 ml of 0.5 mg/ml Derfdrop solution. The 5 mg/kg/d Derfdrop group, each rat was fed with 0.2 ml of 5.0 mg/ml Derfdrop solution. The vehicle or control group was fed with PBS alone. The 1.0 mg/kg/d dexamethasone (Dex) group, each rat was fed with 0.2 ml of 1.0 mg/ml Dex solution. These treatments were performed on 15 consecutive days. The treatment solutions were fed into mouth using a syringe until solutions swallowed.

Derf solution was swallowed, we found the solution was kept in mouth for a few minutes before swallow, therefore, we considered that most of the solution was absorbed by sublingual mucus. Oral administration of Derfdrop solution containing $>55\%$ of Derf1 and $>20\%$ of Derf2 started at 24 h after the last immunization and was continued for 15 consecutive days (Fig. 1). In the 0.5 mg/kg/d Derfdrop group, each 200 g of rat was fed with 0.2 ml of 0.5 mg/ml Derfdrop solution; in the 5 mg/kg/d Derfdrop group, each 200 g of rat was fed with 0.2 ml of 5.0 mg/ml Derfdrop solution; and in the control and vehicle group (Derf-sensitized and vehicle-treated), each rat was fed with same volume of PBS alone. In the 1.0 mg/kg/d dexamethasone (Dex, Jiangsu Henrui Pharmaceutical Co. Ltd., Jiangsu, China) positive control group, each 200 g of rat was fed with 0.2 ml of 1.0 mg/ml Dex solution.

2.5. Assessment of airway hyperresponsiveness

Lung resistance (R_L) and lung dynamic compliance (C_{dyn}) were used to evaluate the changes in airway function after intravenous Mch provocation (the tail vein was cannulated and the injection line passed out of the plethysmograph), Mch dosages (0.016 mg/kg to 2 mg/kg, i.v.) were described previously [16]. The assessment of lung function of anesthetized (urethane, 1.5 g/kg, i.p.) and tracheostomized rats was described previously [16]. Briefly, an anesthetized rat was placed supine inside a Plexiglas whole-body plethysmograph. The flow rate was monitored with a Fisher tube connected to the airway in a pressure transducer. Changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer. To measure pleural pressure, a syringe needle (needle gauge: 12) with a multi-hole tip was directly inserted into the pleural cavity (between the fourth and fifth ribs in left lateral thorax) through a port in the connecting tube with a differential pressure transducer. Transpulmonary pressure was calculated as the difference between mouth and pleural pressure. The signals of all pressure transducers were continuously computed (MedLab, Nanjing Biotech Instruments Co. Ltd., Jiangsu, China) by fitting flow, volume, and pressure to an equation of motion. Changes in R_L and C_{dyn} in response to Mch provocation were analyzed by using a dose–response curve from which the PC_{100} and PC_{25} values for Mch (the provocative concentration of Mch to induce a 100% increase in R_L and to induce a 25% decrease in C_{dyn} by 25%) were calculated.

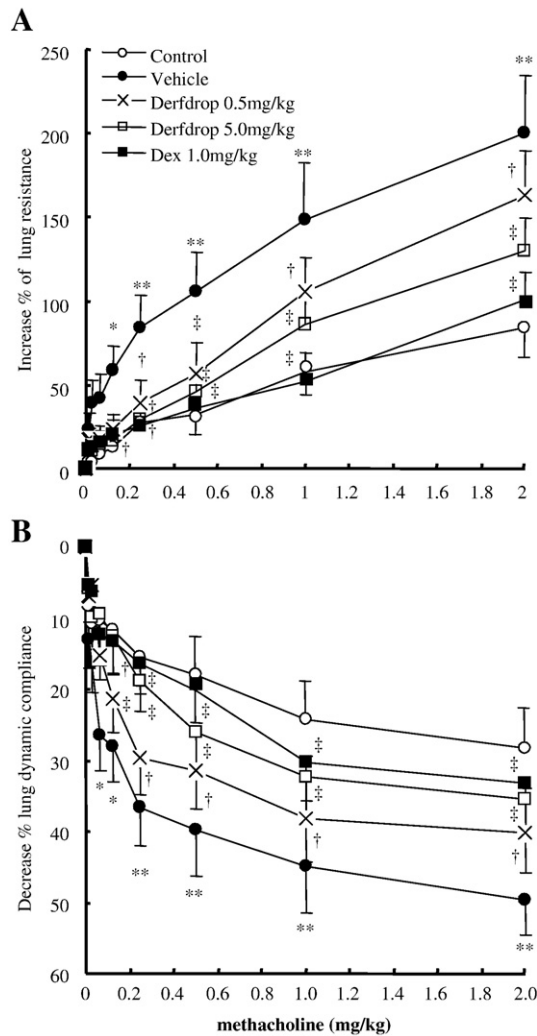


Fig. 2. Dose-dependent response of R_L and C_{dyn} to methacholine. The animals were treated as described in Fig. 1, and 24 h after last Derf allergen challenge, R_L and C_{dyn} against inhalation of methacholine was determined as described in Materials and methods. Data are expressed as mean $\pm$ SD. $n=10$ in each group. * $P<0.05$, ** $P<0.01$ vs control group; † $P<0.05$, ‡ $P<0.01$ vs vehicle group.

2.6. Preparation of bronchoalveolar lavage fluids

Seven days after the challenge, rats were anaesthetized with urethane (1.5 g/kg, i.p.), and the trachea of each rat was surgically exposed and cannulated. The left lung was subjected to the paraffin sections and determination of cytokine levels, however, the right lung was lavaged three times with a single volume of warm PBS (5 ml/kg) containing 1% bovine serum albumin and 5000 IU/l heparin. Cells from BALF were centrifuged once with PBS containing 2% fetal calf serum at $500\times g$ for 10 min at 4 °C. The supernatant was stored at 80 °C until measurement of cytokines. The pellets from BALF were resuspended in PBS, and the Giemsa's staining was performed for classification of leukocytes. The results were expressed as the number of each cell population in 1 L of BALF.

2.7. Measurement of cytokine levels in BALF and lung

IFN- γ and IL-4, which are closely associated with the development of allergic diseases and are used as a representative of Th1 and Th2 cytokines, respectively, were measured by ELISA. Frozen lungs were thawed and cut into small cubes, 100 mg of lung tissues were homogenized in ice-cold PBS buffer (pH 7.4). The BALF (stored at -80 °C) and lung homogenates were centrifuged at $2000\times g$ for 20 min at 4 °C. The concentrations of IFN- γ

and IL-4 in supernatants of BALF and lung homogenates were measured by sandwich ELISA according to the manufacturer's instructions. The limits of IFN- γ (eBioscience Co. San Diego, CA) and IL-4 (U-Cy Tech. bioscience, Netherlands) ELISA kits were 12.5 pg/ml and 10 pg/ml, respectively.

2.8. Determination of serum total IgE and IgG

Total serum IgE and IgG levels were measured by a sandwich ELISA (Bethyl Laboratories, Inc. Montgomery, TX) according to the manufacturer's instruction. Briefly, blood was collected 24 h after the last antigen challenge. Rats were anaesthetized with urethane (1.5 g/kg, i.p.) and bled by femoral artery, and serum was prepared and kept at -80 °C until measurement. Briefly, plates were coated with purified anti-rat IgE and anti-rat IgG (Bethyl Laboratories, Inc.) washed and blocked, and then diluted serum (1:25) was added. Biotinylated anti-rat IgE (Bethyl Laboratories, Inc.) was used for detection of antibody. Purified rat IgE and IgG kappa was used as the standards. After incubation with substrate (OPD), plates were read using an ELISA reader (BIO-TEK, ELX800, Winooski, VT).

2.9. Allergen-specific serum IgE and IgG measurement

Allergen-specific serum IgE and serum IgG levels were measured by ELISA. Briefly, to determine the Derf-specific IgE, 96-well plates were coated with 100 μ l/well of mouse anti-rat IgE heavy chain antibody (Serotec Ltd., Oxford, UK) at a concentration of 2.5 μ g/ml. The next day, after the plates were blocked and washed, 100 μ l of each serum sample (1:5 in blocking buffer) were applied. After an overnight incubation, the plates were treated subsequently with 100 μ l/well of biotinylated Derf allergen (2 μ g/ml) prepared by using Sulfo-NHS-LC-Biotinylation kit (Pierce Biotechnology, Rockford, IL) and horseradish peroxidase-streptavidin (1:1500, Sigma, St. Louis, MO) for 1 h each, at room temperature. For the allergen-specific serum IgG assay, 96-well plates were coated with 100 μ l of purified Derf allergen containing >75% Derf1 antigen (Greer Laboratories, Lenoir, NC) at a concentration of 1.6 μ g/ml in coating buffer and incubated overnight at 4 °C. The next day, after the plates were blocked and washed, 200 μ l of blocking buffer were added to each well followed by 2 h incubation at 37 °C. Diluted serum (1:50) was applied (100 μ l/well) and followed by an overnight incubation at 4 °C. Wells were then washed and treated subsequently with 100 μ l of mouse anti-rat biotinylated IgG (1:5000, AbD Serotec, OX, UK) and horseradish peroxidase-streptavidin (diluted 1:1500) for 1.5 h each at 37 °C. Finally, 100 μ l/well TM Blue (Sigma, St. Louis, MO) was applied as a substrate for horseradish peroxidase, and reactions were allowed to develop at room temperature for at least 10 min. Plates were read at 650 nm by an ELISA plate reader (BIO-TEK).

2.10. Histopathology

The partial left upper lobe was fixed with 10% neutral formalin and the paraffin sections were prepared by histology core at Medical

Table 1

Effects of Derfdrop and Dex on airway hyperresponsiveness to methacholine in asthma model of rats

Groups	n	R_L PC ₁₀₀ (95% CI) Methacholine (mg/kg)	C_{dyn} PC ₂₅ (95% CI) Methacholine (mg/kg)
Control	10	2.25(1.64–3.85)	1.53(0.81–2.89)
Vehicle	10	0.42(0.30–0.57)†	0.07(0.06–0.09)†
Derfdrop (0.5 mg/kg)	10	0.85(0.66–1.10)*	0.22(0.18–0.27)*
Derfdrop (5.0 mg/kg)	10	1.52(1.08–2.13)**	0.47(0.37–0.63)**
Dex (1 mg/kg)	10	1.99(1.36–3.26)**	0.66(0.47–0.91)**

Twenty-four hours after last challenge of allergen, animals were subjected to determination of R_L and C_{dyn} , the Parameters of airway hyperresponse. Methacholine PC₁₀₀ (lung resistance) and PC₂₅ (dynamic lung compliance) were listed, CI: confidence interval. †, $p<0.01$ vs control group; *, $p<0.05$, **, $p<0.01$ vs vehicle group.

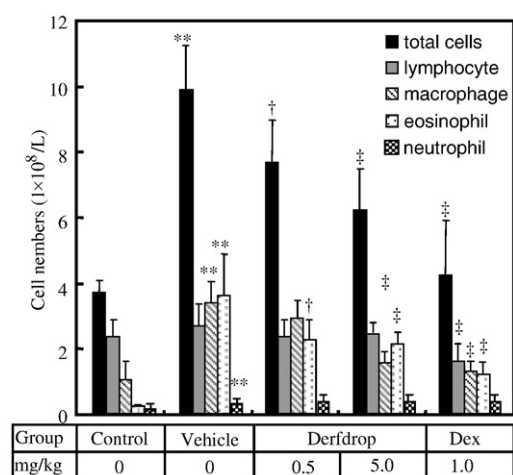


Fig. 3. Effects of Derfdrop and dexamethasone on inflammatory cells accumulation in BALF of allergic rat model. Experimental procedures were described in Fig. 1, twenty-four hours after final challenge with allergen, animals were subjected to harvesting the BALF, total inflammatory cells number and each population of inflammatory cells including eosinophils, lymphocytes, macrophages and neutrophils numbers were counted. Data are presented as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ vs control; † $P < 0.05$, ‡ $P < 0.01$ vs vehicle.

School of Zhejiang University. Alcian blue-periodic acid-Schiff staining (Sigma, St. Louis, MO) for evaluation of mucus production, and H&E staining for examination of cell infiltration were performed. To evaluate the severity of mucus production, Goblet cell hyperplasia in the airway epithelium was blindly semi-quantified under light microscope ($\times 20$ magnification) by using a 5 point grading system described previously [17]. Alcian blue-stained Goblet cells were counted and expressed as the percentage of the total numbers of epithelial cells, and the grading system was described as follows: 0, no Goblet cells; 1, $< 25\%$; 2, 25–50%; 3, 50–75%; 4, $> 75\%$. To determine the severity of inflammatory cell infiltration, peribronchial and perivascular eosinophil infiltration was evaluated blindly based upon a 5 point scoring system described previously [18]; 0, no cells; 1, a few cells; 2, a ring of cells as deep as 1 cell layer; 3, a ring of cells as deep as 2–4 cells; 4, a ring of cells deeper than 4 cells. Scoring of Goblet cells and inflammatory cells was performed in at least three different fields for each lung section. The average scores were obtained from eight animals.

2.11. Statistical analysis

Numerical data are presented as means $\pm$ SD from 6–10 animals per group. Statistics was performed by using SPSS software (version 10.0, SPSS Inc., Chicago, IL, USA), ANOVA and Student *t*-test were used for calculating the difference between each group. Significance was assessed at the $p < 0.05$ level. Experiments were performed independently at least twice and results were qualitatively identical, and representative results are shown.

3. Results

3.1. Allergen-induced airway hyperresponsiveness to methacholine

After subcutaneous sensitization and consecutive 7-d allergen challenge, lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were measured before and after different concentrations of Mch provocation. Rats from vehicle group developed a severe AHR in response to Mch, exhibiting significantly lower values in the R_L of PC₁₀₀ (95% confidence interval, 95% CI) and also significantly lower in the C_{dyn} of PC₂₅ (95% CI) as compared to the control group (Fig. 2 and Table 1). However, Derfdrop or dexamethasone treatment significantly reduced AHR in response to Mch, exhibiting significant increases in PC₁₀₀ and PC₂₅ as compared to the

vehicle treatment. For R_L of PC₁₀₀, the provocative concentrations in response to Mch after treatment with either Derfdrop at 0.5 mg/kg and 5 mg/kg or dexamethasone at 1 mg/kg exhibit 2-, 3- and 4-fold greater than that in vehicle group (each $p < 0.05$), respectively. For C_{dyn} of PC₂₅, the provocative concentrations in response to Mch after treatment with either Derfdrop at 0.5 mg/kg and 5 mg/kg or dexamethasone at 1 mg/kg show 4-, 6- and 9-fold greater than that in vehicle group (each $p < 0.05$), respectively (Fig. 2 and Table 1).

3.2. Inflammatory cells accumulation in the BALF

Allergic asthma is characterized by inflammatory cell accumulation in the airway and lung. To observe the effects of Derfdrop on inflammatory cell accumulation, after Giemsa's staining, inflammatory cell counting and classification were performed. Total cell number in the BALF of vehicle group is greatly higher than that of control group, and each population of inflammatory cells including eosinophils and macrophages in the BALF of vehicle group is significantly higher than those in control group (Fig. 3). Most importantly, eosinophils and macrophages in BALF from the vehicle group were much more robustly increased than lymphocytes, and BALF from the vehicle group contained significantly more eosinophils and

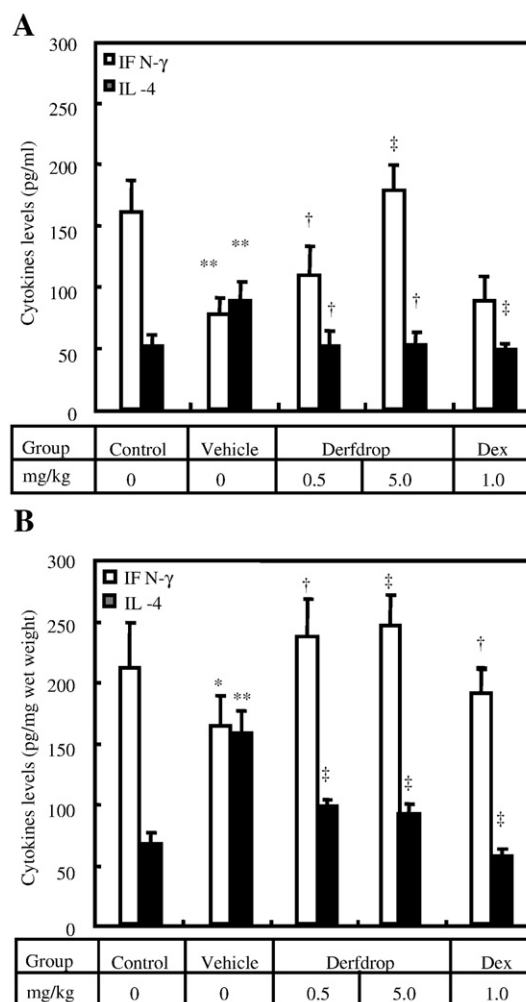


Fig. 4. Effects of Derfdrop and dexamethasone on IFN- γ and IL-4 levels in BALF and lung. The Experiment procedure was described in Fig. 1, BALF and lung tissues were harvested 24 h after the last allergen challenge. IFN- γ and IL-4 levels in BALF (A) and in lung tissues (B) were analyzed by ELISA. Control ($n = 8$), vehicle ($n = 10$), Derfdrop 0.5 mg/kg ($n = 10$), Derfdrop 5 mg/kg ($n = 10$) and dexamethasone 1 mg/kg ($n = 10$). Data are presented as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ vs control group; † $P < 0.05$, ‡ $P < 0.01$ vs vehicle group.

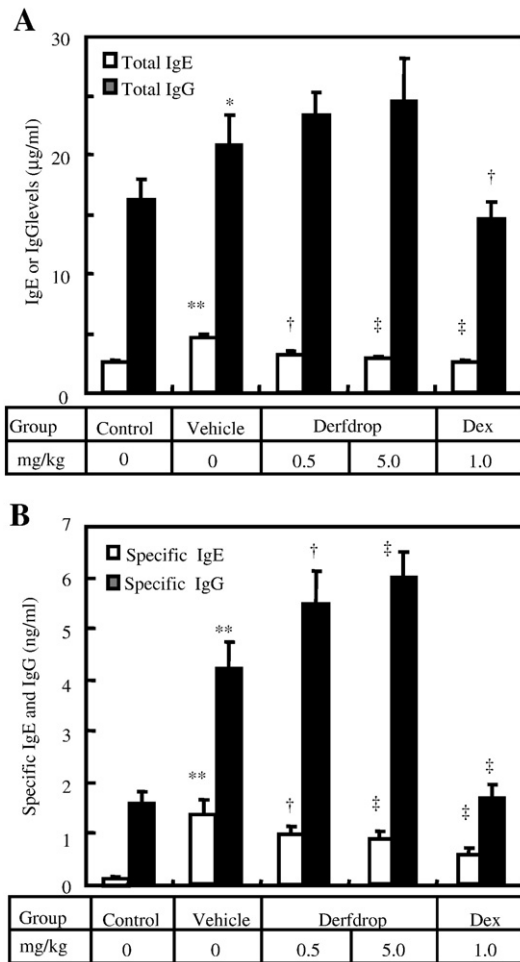


Fig. 5. Effects of Derfdrop and dexamethasone on serum total IgE and IgG as well as allergen-specific IgE and IgG levels. The Experiment procedure was described in Fig. 1, sera were harvest 24 h after the last allergen challenge. Total IgE and IgG (A) as well as allergen-specific IgE and IgG (B) were measured by a sandwich ELISA as described in Materials and methods. Data are presented as means $\pm$ SD and expressed as ng/ml. In total IgE and IgG determination, control ($n=6$), vehicle ($n=8$), Derfdrop 0.5 mg/kg ($n=8$), Derfdrop 5 mg/kg ($n=7$) and dexamethasone 1 mg/kg ($n=6$). In allergen-specific IgE and IgG determination, Control ($n=7$), vehicle ($n=10$), Derfdrop 0.5 mg/kg ($n=10$), Derfdrop 5 mg/kg/d ($n=8$) and dexamethasone 1 mg/kg/d ($n=8$). * $P<0.05$, ** $P<0.01$ vs control; † $P<0.05$, ‡ $P<0.01$ vs vehicle.

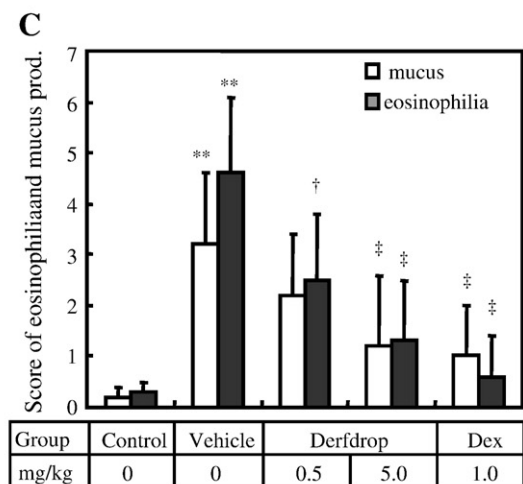
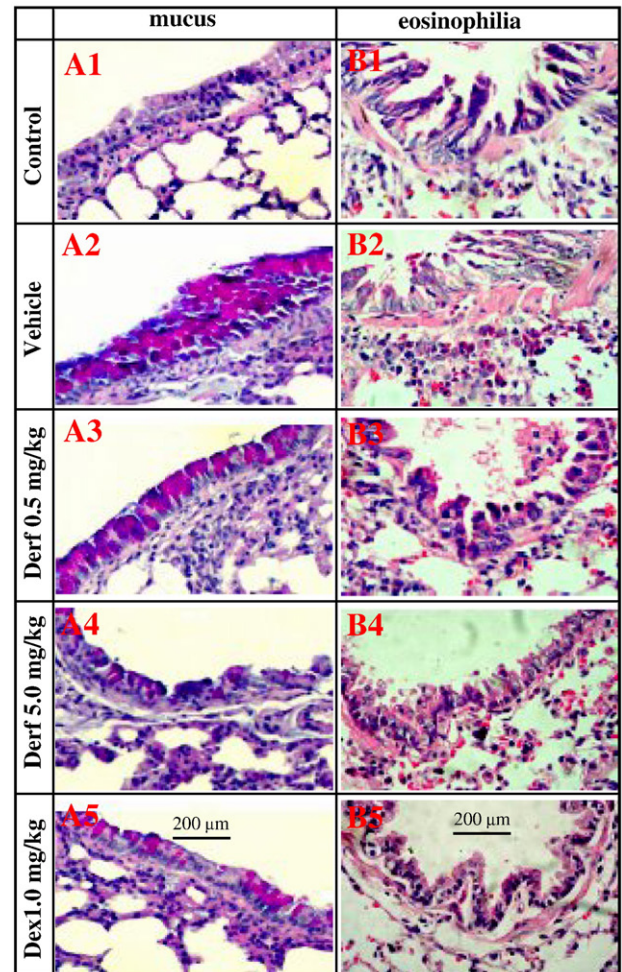
macrophages than lymphocytes. However, treatment with Derfdrop at either 0.5 or 5.0 mg/kg significantly reduced the accumulation of total inflammatory cells as well as eosinophils and macrophages as compared with the vehicle treatment, and the maximal effects were achieved at the dose of 5.0 mg/kg, which is as comparable as dexamethasone treatment at

Fig. 6. Effects of Derfdrop and dexamethasone on lung tissue eosinophilia and mucus production. The experiment procedure was described in Fig. 1, lung tissues were harvested 24 h after the last allergen challenge, and the partial left lobe of the lung was fixed with 10% neutral formalin and sectioned. Alcian blue-periodic acid-Schiff staining for evaluation of mucus production and H&E staining for examination of cell infiltration were performed. Representative images for Goblet hyperplasia and eosinophilia are shown (A and B): A1 and B1, control group; A2 and B2, vehicle group; A3 and B3, Derfdrop 0.5 mg/kg/d; A4 and B4, Derfdrop 5.0 mg/kg/d; A5 and B5, Dex 1.0 mg/kg/d. As described in Materials and methods, to evaluate the severity of mucus production, Goblet cell hyperplasia in the airway epithelium was semi-quantified blindly using a 5 point grading system; to determine the severity of inflammatory cell infiltration, peribronchial and perivascular eosinophil counts were performed blindly based upon a 5 point scoring system. Scoring of Goblet cells and inflammatory cells (C) was performed in at least three different fields for each lung section, data are presented as means $\pm$ SD; $n=8$ in each group. * $P<0.05$, ** $P<0.01$ vs control; † $P<0.05$, ‡ $P<0.01$ vs vehicle.

1 mg/kg (Fig. 3). In the BALF of vehicle group, neutrophil number is about 1% of influx total inflammatory cells, and though neutrophil number is significantly increased in vehicle group as compared with those in control group, treatment with Derfdrop at 0.5 mg/kg/d and 5.0 mg/kg/d or Dex at 1.0 mg/kg/d did not significantly reduced allergen-induced neutrophil accumulation in BALF (Fig. 3).

3.3. Cytokines levels in BALF and lung tissues

BALF and lung tissues were harvested 24 h after the last antigen challenge, Th1 cytokine, IFN- γ , and Th2 cytokine, IL-4, levels were



determined by ELISA, respectively. As shown in Fig. 4, IL-4 levels in both BALF and lung of the vehicle group were significantly increased as compared with the those of control group (both $p < 0.01$), however, IFN- γ levels in both BALF and lung of the vehicle group were significantly decreased as compared with those of control group (both $p < 0.01$). Treatment with Derfdrop at either 0.5 or 5.0 mg/kg significantly inhibited the increases of IL-4 levels in BALF and lung as compared with vehicle treatment (both $p < 0.05$), both of inhibition were as comparable as that of Dex treatment at 1 mg/kg. However, treatment with Derfdrop at either 0.5 or 5.0 mg/kg significantly increased IFN- γ levels in BALF and lung as compared with the vehicle treatment (both $p < 0.05$), both dosages of Derfdrop treatment were more efficient than Dex treatment at 1 mg/kg (Fig. 4).

3.4. Effects of Derfdrop on serum IgE and IgG levels

Among the immunological parameters associated with SLIT, serum IgE and IgG are by far the most important factors related to the immunoresponse. We investigated the effects of Derfdrop on serum total IgE and IgG levels as well as allergen-specific IgE and IgG levels in specific allergen-induced allergic model of rat. Total serum IgE and IgG were measured by ELISA and the results are shown in Fig. 5A. The vehicle group exhibits a significantly higher IgE ($p < 0.01$) and IgG ($p < 0.05$) levels than control group, Derfdrop at either 0.5 mg/kg or 5.0 mg/kg produced a marked decline in total serum IgE levels ($p < 0.05$ and $p < 0.01$, respectively), but did not influence total IgG levels ($p > 0.05$), however, dexamethasone at 1 mg/kg significantly inhibited both total serum IgE and IgG levels ($p < 0.05$ and $p < 0.01$, respectively). Furthermore, we evaluated the potential effects of Derfdrop on the allergen-specific IgE and IgG levels in Derf-induced allergic model of rat. Allergen-specific serum IgG and IgE were measured by ELISA, and the data are shown in Fig. 5B. A significant increase in allergen-specific serum IgE levels in vehicle group was observed as compared with control group, Derfdrop treatment at either 0.5 mg/kg or 5.0 mg/kg dose-dependently and significantly inhibited allergen-specific IgE production ($p < 0.05$ and $p < 0.01$, respectively). Meanwhile, Derfdrop at either 0.5 mg/kg or 5.0 mg/kg significantly elevated serum allergen-specific IgG levels ($p < 0.05$ and $p < 0.01$, respectively), as compared with vehicle group. Furthermore, dexamethasone at 1 mg/kg not only significantly inhibited the elevation of allergen-specific serum IgE levels, but also reduced allergen-specific IgG levels (both $p < 0.05$).

3.5. Effects of Derfdrop on Goblet hyperplasia and eosinophilia in lung of allergic rats

Lung tissue was harvested 24 h after the last antigen challenge, paraffin sections were prepared. Alcian blue-periodic acid-Schiff staining was performed for evaluation of mucus production in airway epithelium, meanwhile, H&E staining was performed for examination of eosinophilia in lung. Vehicle group (Fig. 6A2) developed a marked Goblet cell hyperplasia and mucus hypersecretion (dark pink staining) within the bronchi as compared with control group (Fig. 6A1). Derfdrop at either 0.5 mg/kg (Fig. 6A3) or 5.0 mg/kg (Fig. 6A4) markedly attenuated Goblet cell hyperplasia and hypertrophy as compared with vehicle group, the maximal effects of Derfdrop at 5.0 mg/kg on Goblet cell hyperplasia and hypertrophy are as comparable as those of dexamethasone at 1 mg/kg (Fig. 6A5). Similarly, in vehicle group (Fig. 6B2), Derf allergen aerosol challenge induced severe accumulation of eosinophils into the peribronchi and perivessle as compared with control group (Fig. 6B1); Derfdrop at either 0.5 mg/kg (Fig. 6B3) or 5.0 mg/kg (Fig. 6B4) markedly reduced or inhibited eosinophil-rich leukocyte accumulation as compared with vehicle treatment. The maximal effects of Derfdrop at 5.0 mg/kg on eosinophil accumulation are as comparable as that of dexamethasone at 1 mg/kg (Fig. 6B5). Furthermore, semi-quantitative analyses revealed that Derfdrop treatment at either 0.5 mg/kg or 5 mg/kg significantly attenuated

Derf allergen-induced mucus secretion and the eosinophil-rich leukocyte infiltration in the lungs of sensitized rats, the maximal effects of Derfdrop at 5.0 mg/kg was as comparable as those of dexamethasone treatment at 1 mg/kg (Fig. 6C).

4. Discussion

Sublingual immunotherapy (SLIT), methodologically, the allergen extract is kept under the tongue for a few minutes and then swallowed: this is the so-called 'sublingual-swallow' [11]. In fact, it is hard to submit animals to the SLIT, in the present study, we imitated clinical SLIT by feeding Derfdrop solution into mouth using a syringe until swallowed, and we found the solution was kept in mouth for a few minutes before swallow, therefore, we considered that partial allergen was absorbed by sublingual mucus and partial allergen was absorbed by intestine mucus. Although generally clinical SLIT takes very long time, in our allergen-sensitized rats, feeding Derfdrop in such a short period effectively suppressed specific allergen-induced airway Goblet cell hyperplasia, airway inflammation, AHR, increases of IL-4 and decreases of IFN- γ in the BALF and lung tissues, indicating that our administration system imitating the clinical SLIT could be effective and applicable in the Derf allergen-induced rat model of asthma. Additionally, we did not find any obvious side-effect of Derfdrop administered repeatedly at those dosages, and neither significant changes in body weight nor macroscopic and/or histological alteration of intestine were found after administration of Derfdrop.

The conventionally used allergens include house mite allergen [19–22], pollen extract [23,24], ovalbumin [25] and peanut protein [26]. Many studies showed that oral administration of allergen, including recombinant Derf2, effectively suppresses IgE responses in the allergic model of mice [19,20]. Although we supposed that the effects of Derfdrop is antigen-specific and it is not likely that Derfdrop affects immune response via antigen-non-specific way, it is still necessary to set up the experiments to verify the specificity of Derf allergen by using other allergen-induced asthmatic animal model. Our data indicated that oral administration of Derf extracts effectively attenuated specific allergen-induced IgE response, including airway Goblet cell hyperplasia, inflammatory cell accumulation, and AHR, the maximal effects are as comparable as Dex at 1 mg/kg. We suggested that the effects of Derf extracts on specific allergen-induced IgE response were achieved by inhibition of IgE and elevation of allergen-specific IgG production (Fig. 5), since the shift in balance between IgE and IgG is a crucial evidence for successful allergen-specific immunotherapy [27]. It was believed that one mechanism by which immunotherapy suppresses the allergic response is by an increase in the production of IgG antibodies, primarily the IgG4, over IgE [27]. Because the production of IgE against antigen provocation is the hallmark of allergic responses, and the production of antigen-specific IgG antibodies, can antagonize the allergic inflammation cascade resulted from antigen recognition by IgE [27]. Most of studies indicated that allergen-specific IgE levels are either increased or remain unaffected by SLIT treatment, consistent with our present data, the allergen-specific IgG levels, which are considered to reflect a modulation of allergic Th2-response, are increased in patients actively treated with SLIT [15]. In our present data, The treatments of Dex decreased the production of IgG but Derfdrop enhanced it (Fig. 5), this might be due to the immunosuppressive effect of Dex on B lymphocytes.

Derfdrop significantly increased allergen-specific IgG levels in our experimental system, suggesting that SLIT by Derfdrop may work by modulating the Th2-biased allergic immunoresponse, however, the detailed mechanism by which oral administration of allergen regulates IgE response in allergen-sensitized animals is not fully understood. That regulatory T cells produce transform growth factor (TGF)- β and IL-10 is believed to play an important role in SLIT, T cell IL-10 is a potent suppressor of both total and allergen-specific IgE, while it simultaneously increases IgG4 production [28]. Meanwhile, TGF- β induces IgA production [28]. The role of IgA and TGF- β , as well as IgG4 and IL-10, in peripheral mucosal immune responses to allergens through oral

mucosal dendritic cells, and the balance among IgE, IgG and IgA is critical to the successful outcome of SLIT [28]. In our experimental system, whether oral administration of Derfdrop attenuates IL-10-mediated IgE and IgG production and TGF- β -mediated IgA production is still unknown and deserves further study.

The Th2 cytokines such as IL-4, IL-5 and IL-13, play an important role in the pathogenesis of allergic asthma, and the imbalance between IL-4 and Th1 cytokine, IFN- γ production is widely believed to increase IgE response in allergic individuals. Excessive IgE production is thought to be critical in the pathogenesis of various allergic diseases. In many clinical studies, the relationship between immunotherapy in allergic diseases and Th1/Th2 bias are greatly concerned in various allergic diseases, several lines of evidence indicate that specific immunotherapy may act by modifying the patterns of cytokines produced by Th cells. IL-4 is likely to be the most important cytokine in elevation of in vivo IgE synthesis against HDM in children with bronchial asthma [29]. Specific immunotherapy improved clinical parameters of allergic diseases including symptom scores and medication use during the treatment, and had a marked effect on increasing the IFN- γ /IL-4 ratio in peripheral blood CD4⁺ T cells, but no significant changes in the IFN- γ /IL-4 ratio in peripheral blood CD8⁺ T cells [30]. Clinical improvement is associated with a reduction in allergen-specific IL-4 production and in allergen-specific serum IgE in allergic individuals receiving high-dose maintenance immunotherapy [31]. HDM immunotherapy results in a decrease in Derp2-specific IFN- γ /IL-4 expression [32]. Allergen-specific IFN- γ is produced at far greater levels from CD8⁺ T cells of HDM-sensitive allergic patients than from non-atopic control individuals, and this level is reduced after specific immunotherapy [33]. In the present study, antigen-induced decreases of IFN- γ and increases of IL-4 were observed in the Derf allergen-sensitized and challenged rats, which is consistent with previous study, indicating that low dose of orally administered antigen down-regulates the T helper type 2-response in a murine model of dust mite hypersensitivity [21]. The Th1/Th2 imbalance are correlated with the changes in serum total IgE and IgG, allergen-specific serum IgE and IgG levels, airway responsiveness and eosinophilia. Thus, in the present study, we suggested that AHR improvement is associated with a regulation in Th1/Th2 balance, a decrease in allergen-specific serum IgE and an increase in allergen-specific serum IgG in the allergic model of rats with oral immunotherapy, which is consistent with previous study, indicating that oral hyposensitization with recombinant Der f2 attenuates immediate airway constriction in a murine allergic model [22].

In conclusion, the present study definitely indicates that oral administration of Derfdrop to sensitized rats reduced the AHR and IgE response, improved Th1/Th2 imbalance, and attenuated Goblet cell hyperplasia and eosinophil-rich leukocyte infiltration in the lung tissues. Therefore, this oral administration effectively imitates the clinical SLIT treatment, and Derfdrop improves specific allergen-induced inflammation and AHR in Derf-sensitized allergic rats, which provides with the rationale for clinical SLIT by using Derfdrop in specific allergen-induced asthma.

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