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Oral administration of allergen extracts from mugwort pollen desensitizes specific allergen-induced allergy in mice

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ABSTRACT

Clinically, sublingual immunotherapy (SLIT) using allergen extracts effectively alleviates the symptoms of allergic rhinitis and asthma. We hypothesized that oral administration of a high-dose of allergen extracts imitates SLIT, which may prevent IgE-related responses in allergic diseases. In the present study, we investigated the effects of oral administration of allergen extracts from mugwort pollen (MP) on allergen-induced inflammation and airway hyperresponsiveness (AHR) in an allergic mouse model. After administration of MPdrop containing Art v 1 and Art v 4 extracts derived from MP specifically in MP-sensitized mice, the effects of MPdrop on AHR, inflammatory cell accumulation, cytokine production in the bronchoalveolar lavage fluid and lung tissue, and serum IgE and IgG levels were investigated. The results indicated that MPdrop not only prevented the AHR in response to methacholine in a dose-dependent manner but also significantly reduced the total serum and allergen-specific IgE levels. All of the maximal effects were achieved at a dose of 100 μ g/(kg d) and were comparable to the effects of dexamethasone at a dose of 0.5 mg/(kg d). Furthermore, oral administration of MPdrop dose-dependently elevated allergen-specific serum IgG2a levels, reduced total and allergen-specific IgE levels and normalized the imbalance between the Th1 cytokine IL-12 and Th2 cytokines IL-4 and IL-5. Finally, oral administration of MPdrop significantly reduced goblet cell hyperplasia and eosinophilia in the MP-sensitized allergic mouse model. These data suggest that MPdrop effectively improves specific allergen-induced inflammation and AHR in MP-sensitized and -challenged mice and provides the rationale for clinical use of MPdrop in the specific allergen-induced asthma.

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1. Introduction

Allergic diseases are the most common inflammatory diseases, and their prevalence and incidence have increased in many developed and developing countries [1]. Inhalation of allergens evokes deleterious immune and inflammatory responses, which further lead to allergic pathology and allergic symptoms [1]. A known risk factor for the development of atopic allergy is exposure and sensitization to the pollens. Pollens are important sources of outdoor allergens associated with asthma and other allergic disorders [2]. The overall prevalence of positive skin prick responses in Chinese individuals is 59.0% for *Dermatophagoides farinae*, 57.6% for *Dermatophagoides pteronyssinus*, 40.7% for *Blomia tropicalis*, 16.1% for American cockroach, 14.0% for dog, 11.5% for *Blatella*

germanica, 11.3% for *Artemisia vulgaris*, 10.3% for cat, 6.5% for *Ambrosia artemisiifolia*, 6.3% for mixed mold I, 4.4% for mixed mold IV, 3.5% for mixed grass pollen and 2.2% for mixed tree pollen [3]. The most common and important outdoor aeroallergens are the pollens from *Artemisia vulgaris* (mugwort) and *Ambrosia artemisiifolia* (ragweed). To date, several clinically important allergens, such as Art v 1, Art v 2, Art v 3 and Art v 4, have been identified in crude mugwort extracts [4–6]. Recombinant Art v 1, Art v 2, Art v 3 and Art v 4 have been generated. They have been shown to exhibit allergic activity in humans and animals comparable with native products prepared from crude mugwort pollen (MP) extracts [6]. Because sensitivity to these allergens is present in 60–80% of patients with MP allergies, Art v 1 and Art v 3 could be valuable tools for the diagnosis and immunotherapy of allergic asthma and rhinitis caused by MP. Unlike symptomatic treatment, allergen-specific immunotherapy both improves symptoms and modifies the natural course of the disease in patients with allergic rhinoconjunctivitis, rhinitis and mild asthma [7–10]. Because of traditional therapies, such as subcutaneous immunotherapy (SCIT), carry the

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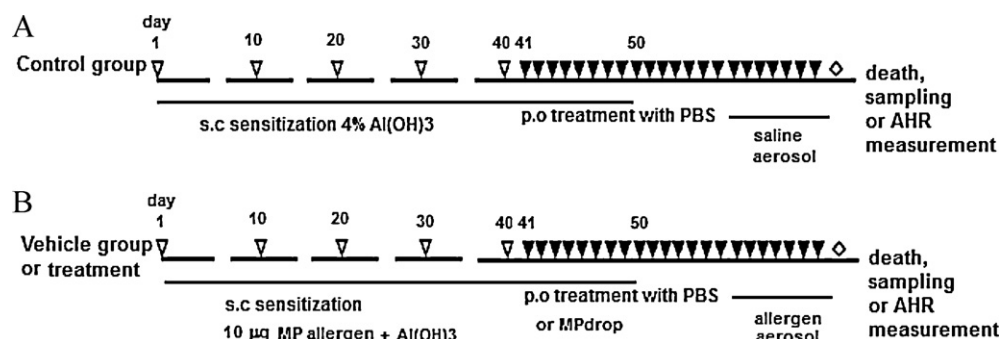


Fig. 1. Experimental procedure. Mice were subcutaneously (s.c.) sensitized five times with 10 µg of MP allergen emulsified in 0.5 ml of 4% aluminum hydroxide adjuvant. Negative control mice (Control group) were injected with 0.5 ml of 4% aluminum hydroxide adjuvant alone following the same protocol. Fifty-six days later, all the animals were placed in a plastic box and challenged via the airways with aerosolized MP allergen (1% in saline) or an equal volume of saline (Control group) in a jet nebulizer for 30 min daily for 7 consecutive days. Oral treatment was initiated at 24 h after the fourth immunization. In the 10 µg/(kg d) MPdrop group, each 20 g of mouse was fed 50 µl of 4 µg/ml MPdrop solution; in the 100 µg/(kg d) MPdrop group, each 20 g of mouse was fed 50 µl of 40 µg/ml MPdrop solution. In the control and vehicle groups (MP antigen-sensitized and vehicle-treated), each mouse was fed the same volume of PBS alone. These treatments were performed on 22 consecutive days. The treatment solutions were fed by mouth with a syringe until the solution was swallowed.

risk of systemic reactions (e.g., anaphylaxis) [11], more convenient alternatives to subcutaneous injections are being developed 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 pollens with rhinoconjunctivitis, rhinitis and asthma [8–12]. However, the detailed effects of MP extracts on the modulation of immunoresponse in allergy are not fully understood. Specifically, questions remain regarding how MP extracts affect IgG and IgE levels in specific allergen-induced asthma and whether MP extracts work by modulating the Th1- or Th2-biased allergic immune response; these questions merit further study [13,14]. Established animal models were utilized to answer these questions [13,14]. In the present study, we used MPdrop solution containing Art v 1 and Art v 3 extracts derived from mugwort (*Artemisia vulgaris*) pollen (MP) as an example of allergen extracts. MPdrop solution was orally administrated to MP-sensitized and -challenged mice. The effects of MPdrop on airway hyperresponsiveness (AHR), cytokine production, inflammatory cell accumulation in the bronchoalveolar lavage fluid (BALF) and lung tissue, and total and allergen-specific IgE and IgG2a levels in serum were investigated. Our data suggested that oral administration of MPdrop effectively improved specific allergen-induced inflammation and AHR in MP-sensitized mice and provide the rationale for clinical use of allergen extracts for the treatment of allergic diseases.

2. Materials and methods

2.1. Animals

Female, 6–8-week-old BALB/c mice were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Certificate No. SCXK 2007-0005, Shanghai, China). All animals were housed in a room maintained at 23 ± 2 °C with 50 ± 10% humidity and a 12-h light, 12-h dark cycle (lights on from 8:00 A.M. to 8:00 P.M.). The animals were allowed free access to tap water and regular rodent chow. Rodent chow was withheld for 8 h prior to the experiments. All the animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. The animals were terminally euthanized by inhalation of CO₂.

2.2. Antigen

Endotoxin-free *Artemisia vulgaris* pollen (lot T20061020) was collected from a good agricultural practice (GAP) base of Zhejiang Wowu Biotech Co., Ltd. (Zhejiang Wowu Biotech Co., Ltd., Hangzhou

City, China). The presence of endotoxin in the *Artemisia vulgaris* pollen was inspected using the limulus agent method. Allergens derived from *Artemisia vulgaris* pollen (MP) were purified by breaking the pollen wall using a mechanical method, defattening with acetone, extraction with 0.125 M ammonium bicarbonate, and further dialysis with distilled water. Additional purification was performed using DEAE ion exchange chromatography and gel filtration, and the preparation of MP extracts (MPdrop solution, lot T20061227) containing >50% of group 1 allergen (Art v 1) and >20% of group 3 allergen (Art v 3) was used for oral administration in mice (sublingual immunotherapy for human). However, the preparation used for antigen-sensitized mice (MP allergen, lot T20061124) contained >80% of Art v 1 and >55% of Art v 3.

2.3. Experimental procedure

The sensitization of the mice were used in the experiments was performed as previously described [14–16]. Briefly, 10 µg of MP allergen (approximately 5.8 µg Art v 1 and 3.1 µg Art v 3) emulsified in 0.5 ml of 4% aluminum hydroxide adjuvant was subcutaneously injected into the footpad, neck, back and groin of each animal, and the injections were performed on days 1, 10, 20, 30, 40 and 50. Negative control mice (control group) were subcutaneously injected with 0.5 ml of 4% aluminum hydroxide adjuvant only. Beginning on day 56, control and sensitized mice were placed in a plastic box and challenged for 30 min daily via the airways with aerosolized MP 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). The AHR to methacholine (Mch, Sigma, St. Louis, MO, USA) was measured 24 h after the last challenge in 5 groups of mice. In another 5 groups of mice, sera were harvested for assessment of immunoglobulin levels, and the BALF was prepared from the right lung to be used for inflammatory cell counting and cytokine measurement. A 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 MPdrop

Because SLIT is difficult to be performed in animals, we imitated clinical SLIT by feeding MPdrop solution into animal mouths using a syringe until the MPdrop solution was swallowed. We found that the solution was held in the mouth for a few minutes before swallowing; therefore, we considered most of the solution have been absorbed by the sublingual mucosa. Oral administration of MPdrop solution started at 24 h after the last immunization and

was continued for 15 consecutive days (Fig. 1). In the 10 µg/(kg d) MPdrop group (10 µg MPdrop approximately contain 3.2 µg Art v 1 and 1.5 µg Art v 3), each 20 g of mouse was fed 50 µl of 4 µg/ml MPdrop solution; in the 100 µg/(kg d) MPdrop group, each 20 g of mouse was fed 50 µl of 40 µg/ml MPdrop solution. In the control and vehicle groups (MP antigen-sensitized and vehicle-treated), each mouse was fed the same volume of PBS alone. In the 0.5 mg/(kg d) dexamethasone (Dex, Jiangsu Henrui Pharmaceutical Co., Ltd., Jiangsu, China) positive control group, each 20 g of mouse was fed 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 (0.016–2 mg/kg, i.v.) have been previously described [17–19]. The assessment of lung function of anesthetized (urethane, 1.5 g/kg, i.p.) and tracheostomized mice has been previously described [17–19]. Briefly, an anesthetized mouse was placed supine inside a Plexiglas whole-body plethysmograph. The mouse flow 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 the 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 the 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 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 a 25% decrease in C_{dyn}) were calculated.

2.6. Preparation of bronchoalveolar lavage fluids

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

2.7. Measurement of cytokine levels in BALF and the lung

IFN-γ, IL-12, IL-4 and IL-5, which are closely associated with the development of allergic diseases and used as representative of Th1 and Th2 cytokines, were measured by ELISA. Frozen lungs were thawed and cut into small cubes, and 100 mg of lung tissues were homogenized in ice-cold PBS buffer (pH 7.4). The lung homogenates were centrifuged at 2000 × g for 20 min at 4 °C. The concentrations of IFN-γ, IL-12, IL-4 and IL-5 in the supernatants of lung homogenates were measured by sandwich ELISA, according

to the manufacturer's instructions. The limits of the IFN-γ (eBioscience Co., San Diego, CA) and IL-4 (U-CyTech. Bioscience, the Netherlands) ELISA kits were 12.5 pg/ml and 10 pg/ml, respectively.

2.8. Determination of total serum IgE and IgG

Total serum IgE (Bethyl Laboratories, INC., Montgomery, TX) and IgG levels (Innovative Research, INC., Michigan) were measured using the ELISA kits, according to the manufacturer's instruction. Briefly, blood was collected 24 h after the last antigen challenge. The mice were anaesthetized with ether and bled from the orbital artery, and serum was prepared and stored at –80 °C until measurement. Briefly, the plates were coated with purified anti-mouse IgE and anti-mouse IgG, washed and blocked, and diluted serum (1:25) was then added. Biotinylated anti-mouse IgE was used for detection of the antibody. Purified mouse IgE and IgG kappa were used as the standards. After incubation with the substrate (OPD), the plates were read using an ELISA reader (BioTek, ELX800, Winooski, VM).

2.9. Allergen-specific serum IgE and IgG measurements

Allergen-specific serum IgE and serum IgG levels were measured by ELISA. Briefly, to determine the MP-specific IgE, 96-well plates were coated with 100 µl/well of rat anti-mouse IgE heavy chain antibody (BD Biosciences Pharmingen, San Diego, CA, USA) at a concentration of 2.5 µg/ml. The next day, after the plates were blocked and washed, 100 µl of each serum sample (1:5 in blocking buffer) was applied. After an overnight incubation, the plates were subsequently treated with 100 µl/well of biotinylated MP allergen (2 µg/ml), which was prepared using the 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 IgG2a assay, 96-well plates were coated with 100 µl of purified MP allergen containing >80% Art v1 at a concentration of 1.6 µg/ml in coating buffer and incubated overnight at 4 °C. The next day, after the plates were blocked and washed, 200 µl of blocking buffer was added to each well, followed by 2 h incubation at 37 °C. Diluted serum (1:50) was applied (100 µl/well) and incubated overnight at 4 °C. The wells were then washed and subsequently treated with 100 µl of biotinylated monoclonal anti-mouse IgG2a antibody (1:5000, BD Biosciences Pharmingen, San Diego, CA, USA) and horseradish peroxidase–streptavidin (diluted 1:1500) for 1.5 h each at 37 °C. Finally, 100 µl/well of TM Blue (Sigma, St. Louis, MO) was applied as a substrate for horseradish peroxidase, and the reactions were allowed to develop at room temperature for at least 10 min. The plates were read at 650 nm by an ELISA plate reader (BioTek).

2.10. Histopathology

A portion of the left upper lobe of the lung was fixed with 10% neutral formalin, and paraffin sections were prepared by the histology core at the Medical School of Zhejiang University. Alcian Blue-Periodic acid-Schiff staining (Sigma, St. Louis, MO) was performed to evaluate mucus production, and H&E staining was performed to examine cell infiltration. To evaluate the severity of mucus production, goblet cell hyperplasia in the airway epithelium was blindly semi-quantified under a light microscope (×20 magnification) using a 5-point grading system as previously described [17]. Alcian blue-stained goblet cells were counted and expressed as the percentage of the total number of epithelial cells, and the grading system was employed 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 blindly evaluated using a 5-point scoring system, as previously described [19]: 0, no cells; 1, a few cells; 2, a ring of

Table 1
Effects of MPdrop on airway hyperresponsiveness to methacholine in mouse asthma model.

Groups	n	R _L PC ₁₀₀ (95% CI) Methacholine (mg/kg)	C _{dyn} PC ₂₅ (95% CI) Methacholine (mg/kg)
Control	9	>0.5	0.49(0.37–1.10)
Vehicle	9	0.08(0.06–0.17)*	0.11(0.08–0.15)*
MPdrop 10 µg/kg	9	0.14(0.09–0.23)	0.15(0.13–0.21)
MPdrop 100 µg/kg	8	0.26(0.19–0.35)*	0.28(0.22–0.34)*
Dex 0.5 mg/kg	10	0.28(0.21–0.38)*	0.26(0.21–0.38)*

Twenty-four hours after the last challenge with allergen, the animals were subjected to the determination of R_L and C_{dyn} , the parameters of airway hyperresponsiveness. Methacholine PC₁₀₀ (lung resistance) and PC₂₅ (dynamic lung compliance) are listed; CI, confidence interval.

$p < 0.01$ vs. control group.
* $p < 0.05$ vs. vehicle group.

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. The 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 the means $\pm$ S.E.M. The statistical analyses were performed using SPSS software (version 11.0, SPSS Inc., Chicago, IL, USA). ANOVA and Student's t -tests were used for calculating the differences between each group. Significance was assessed at the $p < 0.05$ level. All the experiments were performed independently at least twice, and the results were qualitatively identical. 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 provocation with different concentrations of Mch. Mice from the vehicle group developed a severe AHR in response to Mch, exhibiting significantly lower values in R_L of PC₁₀₀ (95% CI) and in C_{dyn} of PC₂₅ (95% CI) compared with the control group (Fig. 2 and Table 1). However, MPdrop or Dex treatment significantly reduced the AHR in response to Mch, and significant increases in PC₁₀₀ and PC₂₅ were observed compared with the vehicle treatment group. For the R_L of PC₁₀₀ and C_{dyn} of PC₂₅, the provocative concentrations in response to Mch after treatment with either MPdrop at 100 µg/kg or Dex at 0.5 mg/kg were both approximately 2-fold greater than that in the vehicle group (each $p < 0.05$) (Fig. 2 and Table 1).

3.2. Accumulation of inflammatory cells in the BALF

Allergic asthma is characterized by inflammatory cell accumulation in the airway and lung. To observe the effects of MPdrop on inflammatory cell accumulation, inflammatory cell counting and classification were performed in Giemsa-stained samples. The total cell number in the BALF of the vehicle group was significantly higher than that of control group. Each population of inflammatory cells, including eosinophils and macrophages, in the BALF of the vehicle group was significantly larger than in the control group (Fig. 3). Most importantly, the increase in eosinophils and macrophages in BALF from the vehicle group was much more robust than that of lymphocytes. Interestingly, the BALF from the vehicle group contained significantly more eosinophils and macrophages than lymphocytes. However, treatment with either 10 µg/kg or 100 µg/kg MPdrop significantly reduced the accumulation of total

inflammatory cells, eosinophils and macrophages compared with the vehicle treatment. The maximal effects were achieved at the dose of 100 µg/kg, which was comparable to Dex treatment at 0.5 mg/kg (Fig. 3). In the BALF from the vehicle group, the neutrophil number was approximately 1% of the total influxed inflammatory cells. Although the number of neutrophils was significantly higher in the vehicle group than in the control group, treatment with

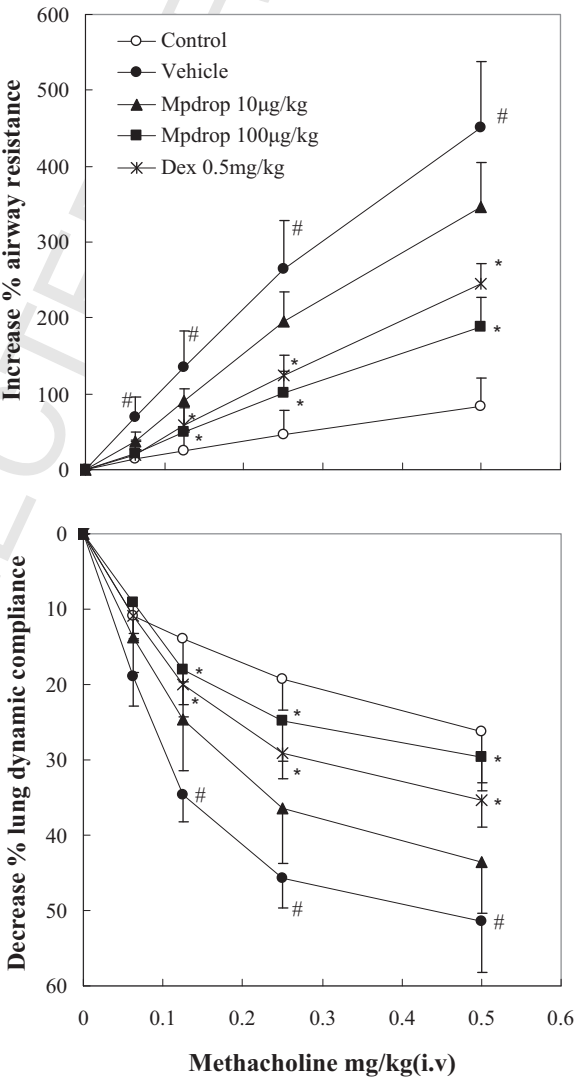


Fig. 2. Dose-dependent response in R_L and C_{dyn} to methacholine. The animals were treated as described in Fig. 1, and 24 h after the last allergen challenge, R_L and C_{dyn} against intravenous administration of methacholine was determined, as described in Section 2. The data are expressed as the mean $\pm$ S.E.M. $n = 7$ –10 in each group. # $p < 0.05$ vs. control group; * $p < 0.05$ vs. vehicle group.

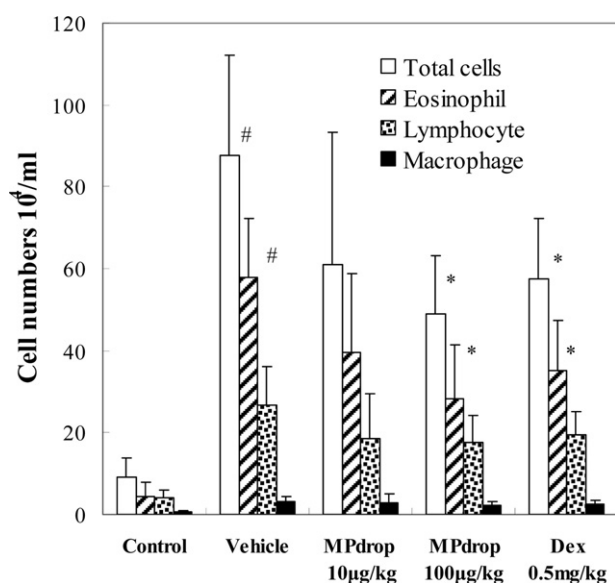


Fig. 3. Effects of MPdrop on inflammatory cell accumulation in BALF from the allergic mouse model. The experimental procedures were described in Fig. 1; twenty-four hours after the final challenge with allergen, the animals were subjected to BALF harvest, and the total numbers of inflammatory cells and of each population of inflammatory cells, including eosinophils, lymphocytes and macrophages, were counted. The data are presented as the means $\pm$ S.E.M. # $p < 0.05$ vs. control; * $p < 0.05$ vs. vehicle.

MPdrop at 10 $\mu\text{g}/(\text{kg d})$ and 100 $\mu\text{g}/(\text{kg d})$ or Dex at 0.5 $\text{mg}/(\text{kg d})$ did not significantly reduce the allergen-induced neutrophil accumulation in the BALF.

3.3. Cytokine levels in BALF and lung tissues

BALF and lung tissues were harvested 24 h after the last antigen challenge, and Th1 cytokine IFN- γ and IL-12 and Th2 cytokine IL-4 and IL-5 levels were determined by ELISA. As shown in Fig. 4, IL-4 and IL-5 levels in the lungs of the vehicle group were significantly higher than in the control group (both $p < 0.05$); however, the IL-12 level, but not the IFN- γ level, in the lungs of the vehicle group was significantly lower compared with the control group ($p < 0.05$). Treatment with either 10 or 100 $\mu\text{g}/\text{kg}$ MPdrop significantly

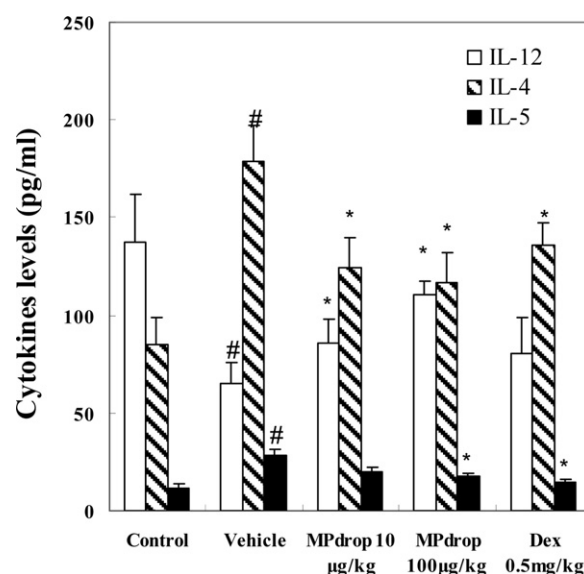


Fig. 4. Effects of MPdrop on cytokine levels in the lung. The experimental procedure was described in Fig. 1; lung tissues were harvested 24 h after the last allergen challenge. The cytokine levels in the lung tissues were analyzed by ELISA. Control ($n = 8$), vehicle ($n = 10$), MPdrop 10 $\mu\text{g}/\text{kg}$ ($n = 10$), MPdrop 100 $\mu\text{g}/\text{kg}$ ($n = 10$), and dexamethasone 0.5 mg/kg ($n = 10$) are shown. The data are presented as the means $\pm$ S.E.M. # $p < 0.05$ vs. control group; * $p < 0.05$ vs. vehicle group.

inhibited the increases in the IL-4 and IL-5 levels compared with vehicle treatment, and the inhibition of both cytokines was comparable to that of Dex treatment at 0.5 mg/kg . However, treatment with MPdrop at either 10 or 100 $\mu\text{g}/\text{kg}$ significantly increased the IL-12 level, but not the IFN- γ level, in the lung compared with the vehicle treatment ($p < 0.05$). Both dosages of MPdrop treatment were more efficient than Dex treatment at 0.5 mg/kg , which had no effect in the test (Fig. 4).

3.4. Effects of MPdrop on IgE and IgG levels in the serum

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 MPdrop on the total IgE and IgG levels in serum as well as allergen-specific

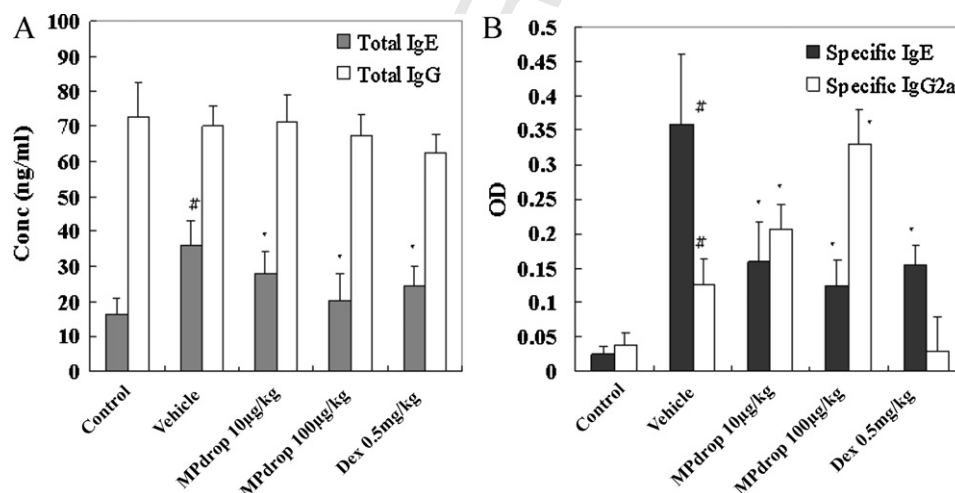


Fig. 5. Effects of MPdrop on total serum IgE and IgG and on allergen-specific IgE and IgG2a levels. The experimental procedure was described in Fig. 1; the serum was harvested 24 h after the last allergen challenge. The total IgE and IgG (A) and the allergen-specific IgE and IgG2a (B) were measured by a sandwich ELISA, as described in Section 2. The data are presented as the means $\pm$ S.E.M. and expressed as ng/ml or as an OD value. For the total IgE and IgG determination, control ($n = 10$), vehicle ($n = 9$), MPdrop 10 $\mu\text{g}/\text{kg}$ ($n = 9$), or MPdrop 100 $\mu\text{g}/\text{kg}$ ($n = 10$) and dexamethasone 0.5 mg/kg ($n = 9$) were collected. For the allergen-specific IgE and IgG2a determination, control ($n = 8$), vehicle ($n = 10$), MPdrop 10 $\mu\text{g}/\text{kg}$ ($n = 10$), MPdrop 100 $\mu\text{g}/\text{kg}$ ($n = 8$) and Dex 0.5 mg/kg ($n = 8$) were collected. # $p < 0.05$ vs. control; * $p < 0.05$ vs. vehicle.

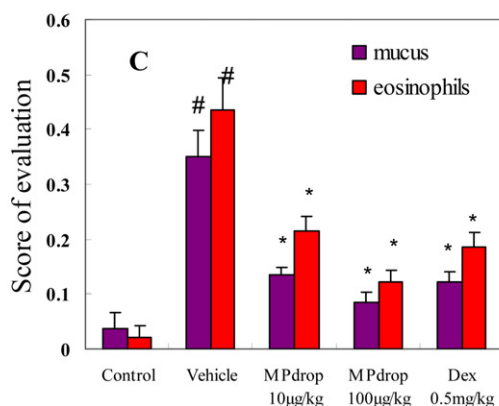
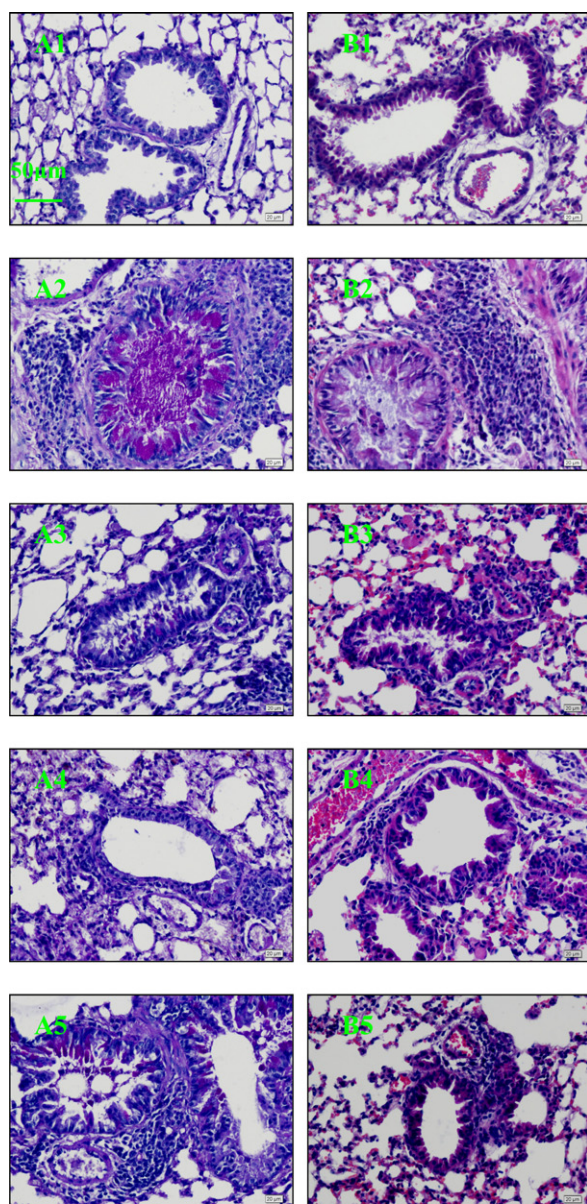


Fig. 6. Effects of MPdrop on lung tissue eosinophilia and mucus production. The experimental procedure was described in Fig. 1; lung tissues were harvested 24 h after the last allergen challenge, and a portion of the left lobe of the lung was fixed with 10% neutral formalin and sectioned. Alcian Blue-Periodic acid-Schiff staining was performed to evaluate mucus production, and H and E staining was performed to examine cell infiltration. 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, MPdrop 10 µg/kg; A4 and B4, MPdrop 100 µg/kg; A5 and

IgE and IgG2a levels in specific allergen-induced allergic mouse models. The total IgE and IgG levels in serum were measured by ELISA (Fig. 5A). The vehicle group exhibited significantly higher IgE ($p < 0.05$) but not IgG ($p > 0.05$) levels than those of the control group. MPdrop at either 10 µg/kg or 100 µg/kg produced a remarkable decline in the total IgE levels in serum ($p < 0.05$). However, MPdrop did not influence the total IgG levels ($p < 0.05$). In comparison, Dex at 0.5 mg/kg significantly inhibited total IgE in the serum ($p < 0.05$) without influencing the IgG levels. Furthermore, we evaluated the potential effects of MPdrop on the allergen-specific IgE and IgG2a levels in the MP-induced allergic mouse model. Allergen-specific IgG2a and IgE levels in serum were measured by ELISA (Fig. 5B). A significant increase in allergen-specific serum IgE and IgG2a levels was observed in the vehicle group compared with the control group. MPdrop treatment at either 10 µg/kg or 100 µg/kg significantly inhibited allergen-specific IgE and increased IgG2a production in a dose-dependent manner ($p < 0.05$). Furthermore, Dex at 0.5 mg/kg significantly inhibited both the elevation of allergen-specific IgE levels in serum and allergen-specific IgG2a levels (both $p < 0.05$).

3.5. Effects of MPdrop on goblet hyperplasia and eosinophilia in the lungs of allergic mice

Lung tissue was harvested 24 h after the last antigen challenge, and paraffin sections were prepared. Alcian Blue-Periodic acid-Schiff staining was performed to evaluate mucus production in the airway epithelium. Meanwhile, H&E staining was performed to assess eosinophilia in the lung. The vehicle group (Fig. 6A2) developed remarkable goblet cell hyperplasia and mucus hypersecretion (dark pink staining) within the bronchi compared with the control group (Fig. 6A1). MPdrop at either 10 µg/kg (Fig. 6A3) or 100 µg/kg (Fig. 6A4) markedly attenuated the goblet cell hyperplasia and hypertrophy compared with the vehicle group. The effects of MPdrop at 100 µg/kg on goblet cell hyperplasia and hypertrophy were comparable to those of Dex at 0.5 mg/kg (Fig. 6A5). Similarly, in the vehicle group (Fig. 6B2), MP allergen aerosol challenge induced a severe accumulation of eosinophils into the peribronchi and perivessle (Fig. 6B1); MPdrop at either 10 µg/kg (Fig. 6B3) or 100 µg/kg (Fig. 6B4) markedly reduced the eosinophil-rich leukocyte accumulation compared with the vehicle treatment. The effects of MPdrop at 100 µg/kg on the inhibition of eosinophil accumulation were comparable with those of Dex at 0.5 mg/kg (Fig. 6B5). Furthermore, semi-quantitative analyses revealed that MPdrop treatment either 10 µg/kg or 100 µg/kg significantly attenuated the MP allergen-induced mucus secretion and eosinophil-rich leukocyte infiltration in the lungs of sensitized mice, and the effects of MPdrop at 100 µg/kg were comparable to those of Dex treatment at 0.5 mg/kg (Fig. 6C).

4. Discussion

Sublingual immunotherapy (SLIT) is a methodology to administer allergen extracts in which the allergen is held under the tongue for a few minutes and then swallowed; this method is the so-called 'sublingual-swallow' [10,12]. In fact, subjecting animals to SLIT is challenging. In the present study, we used a syringe to

B5, Dex 0.5 mg/kg. As described in Section 2, to evaluate the severity of the mucus production, goblet cell hyperplasia in the airway epithelium was blindly semi-quantified using a 5-point grading system. To determine the severity of inflammatory cell infiltration, peribronchial and perivascular eosinophil counts were blindly performed using a 5-point scoring system. The scoring of goblet cells and inflammatory cells (C) was performed in at least three different fields for each lung section, and the data are presented as the means $\pm$ S.E.M; $n = 9$ in each group. # $p < 0.05$ vs. control; * $p < 0.05$ vs. vehicle.

feed MPdrop solution into the mouths of mice for a few minutes until the solution was swallowed. Therefore, we considered a portion of the allergen have been absorbed by the sublingual mucus and additional allergen absorbed by the intestinal mucus. We admit that applying the mucoadhesive formulations established by Razafindratsita's group would be better and more closely imitates clinical SLIT [13]. Clinical SLIT usually takes a very long time. However, in our allergen-sensitized mice, the short period of feeding MPdrop effectively suppressed specific allergen-induced airway goblet cell hyperplasia, airway inflammation and AHR, and increased IL-4 and decreased IFN- γ levels in both BALF and lung tissues; these data indicate that oral administration can be effective and applicable in the MP allergen-induced mouse model of asthma. Additionally, we did not observe any obvious side effects of repeated MPdrop administration at the tested dosages. Neither significant changes in body weight nor macroscopic and/or histological alterations in the intestine were found after administration of MPdrop. The conventionally used allergens are house mite allergen [20–23], pollen extract [24–26], ovalbumin [27] and peanut protein [28]. Many studies have shown that oral administration of allergens, including pollen allergen, effectively suppresses IgE responses and attenuates allergic symptoms in the allergic patients [20–31]. Although we assumed that the effects of MPdrop were primarily antigen-specific, establishing a MP-specific allergen-induced asthmatic animal model is necessary to verify the specific immunotherapy effect and mechanism of MPdrop. Interestingly, our data indicate that short-term oral administration of MP extracts effectively attenuated the specific allergen-induced IgE response, and the results were consistent with those of other immune therapy approaches in mice [20,27,32,33]. Surprisingly, mice exposed to 1% or 2% ovalbumin (OVA) dissolved in drinking water for 5–7 consecutive days became unresponsive to subsequent immunogenic OVA challenges [20,27]. In contrast, clinical SLIT requires a long time to cure allergic disease by down-regulating the level of IgE [10]. We suspect that this difference may be due to the biological life cycle or species differences. We propose that the effects of MP extracts on the specific allergen-induced IgE response were achieved by inhibition of IgE and elevation of allergen-specific IgG2a production (Fig. 5) because the shift in the balance between IgE and IgG2a is crucial evidence of successful allergen-specific immunotherapy [34]. One mechanism of suppression of the allergic response by immunotherapy is through increasing the production of IgG antibodies, primarily IgG2a antibodies [27]. The inhibitory effects of IgE against antigen provocation are hallmarks of therapy against allergic responses. The production of antigen-specific IgG2a antibodies can antagonize the allergic inflammatory cascades resulting from antigen recognition by IgE [34]. Most studies have indicated that allergen-specific IgE levels either increase or remain unaffected by SLIT treatment, which is consistent with our present data. In contrast, the allergen-specific IgG1 and IgG4 levels in humans (IgG2a in mice), which are considered to reflect a modulation of allergic Th2-response, increase in patients actively treated with SLIT [10,12]. In our present data, treatment with Dex decreased the production of IgG2a, which is opposite to the effects of MPdrop (Fig. 5). This difference might be due to the immunosuppressive effects of Dex on B lymphocytes. MPdrop significantly increased allergen-specific IgG2a levels in our experimental system, suggesting that oral tolerance of MPdrop may be due to the modulation of Th2-biased allergic immunoresponse. The detailed mechanism of regulation of the IgE response by oral administration of specific allergens is not fully understood. IL-12 produced by regulatory T cells is believed to be a potent suppressor of both total and allergen-specific IgE [35,36] while simultaneously increasing IgG2a, IgG2b and IgG3 antibodies [37]. In our experimental system, oral administration of MPdrop enhanced IL-12 but not IFN- γ , which is directly related to both the reduction of

allergen-specific IgE and enhancement of allergen-specific IgG2a levels [35–37].

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 cytokines. IFN- γ production is widely believed to increase the 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 and the Th1/Th2 bias has been of great concern in various allergic diseases. Several studies have indicated that specific immunotherapy may act by modifying the patterns of cytokines produced by Th1 cells. IL-4 is likely to be the most important cytokine in the *in vivo* elevation of IgE synthesis against pollen in children with bronchial asthma [38,39]. Specific immunotherapy has improved the clinical parameters of allergic diseases, including symptom scores and medication use during treatment. IL-4 markedly facilitates the increase in the Th1/Th2 cytokine ratio in peripheral blood CD4 $^{+}$ T cells but not in peripheral blood CD8 $^{+}$ T cells [39,40]. 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 [40]. Pollen immunotherapy results in decreases in IL-4 and IL-13 expression [39]. In the present study, antigen-induced decreases in IL-12 (but not in IFN- γ) and increases in IL-4 and IL-5 were observed in the MP allergen-sensitized and -challenged mice, which is consistent with a previous study that has indicated that a low dose of orally administered antigen down-regulates the T helper type 2-response in a murine model of pollen hypersensitivity [24,25]. The Th1/Th2 imbalance correlates with the changes in the total serum IgE and IgG levels, allergen-specific serum IgE and IgG2a levels, airway responsiveness and eosinophilia. Thus, in the present study, we suggested that the AHR improvement is associated with regulation of the Th1/Th2 balance, a decrease in allergen-specific serum IgE and an increase in allergen-specific serum IgG2a in the allergic mouse model upon treatment with oral immunotherapy. Our data are consistent with a previous study that has indicated that oral hyposensitization with recombinant pollen allergen attenuates the immediate airway constriction in a murine allergic model [24,25].

In conclusion, the present study demonstrated that oral administration of MPdrop reduced the AHR and IgE responses, improved the Th1/Th2 imbalance and attenuated the goblet cell hyperplasia and eosinophil-rich leukocyte infiltration in the lung of sensitized mice. This approach effectively imitated clinical SLIT treatment. MPdrop attenuated specific allergen-induced inflammation and AHR in MP-sensitized allergic mice, which provides rationale for the use of MPdrop in clinical SLIT for the treatment of specific allergen-induced asthma.

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