Effect of *Zedoariae rhizoma* on Bronchial Inflammation and Allergic Asthma in Mice

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There are detailed descriptions of the clinical experiences and prescriptions of asthma in traditional Korean medicine. *Zedoariae rhizoma* is one of the Korean herbal medicines used to treat bronchial asthma and allergic rhinitis for centuries. However, the therapeutic mechanisms of this medication are still far from clear. In this study, a house-dust-mite (Dermatophagoides pteronyssinus [Der p])-sensitized murine model of asthma was used to evaluate the immunomodulatory effect of *Zedoariae rhizoma* on the allergen-induced airway inflammation in asthma. Three different protocols were designed to evaluate the treatment and/or long-term prophylactic effect of *Zedoariae rhizoma* in Der p-sensitized mice. Cellular infiltration and T-cell subsets in the bronchoalveolar lavage fluid (BALF) of allergen-challenged mice were analyzed. Intrapulmonary lymphocytes were also isolated to evaluate their response to allergen stimulation. When *Zedoariae rhizoma* was administered to the sensitized mice before AC (groups A and C), it suppressed airway inflammation by decreasing the number of total cells and eosinophil infiltration in the BALF, and downregulated the allergen- or mitogen-induced intrapulmonary lymphocyte response of sensitized mice as compared to those of controls. This immunomodulatory effect of *Zedoariae rhizoma* may be exerted through the regulation of T-cell subsets by elevation or activation of the CD8+ and double-negative T-cell population in the lung. However, the administration of *Zedoariae rhizoma* to sensitized mice 24 h after AC (group B) did not have the same inhibitory effect on the airway inflammation as *Zedoariae rhizoma* given before AC. Thus, the administration of *Zedoariae rhizoma* before AC has the immunomodulatory effect of reducing bronchial inflammation in the allergen-sensitized mice. On the other hand, to determine the potentiality of prophylactic and/or therapeutic approaches using a traditional herbal medicine, *Zedoariae rhizoma*, for the control of allergic disease, we examined the effects of oral administration of *Zedoariae rhizoma* on a murine model of asthma allergic responses. When oral administration of *Zedoariae rhizoma* was begun at the induction phase immediately after OVA sensitization, eosinophilia and Th2-type cytokine production in the airway were reduced in OVA-sensitized mice following OVA inhalation.These results suggest that the oral administration of *Zedoariae rhizoma* dichotomously modulates allergic inflammation in a murine model for asthma, thus offering a different approach for the treatment of allergic disorders.

Key words : *Zedoariae rhizoma*, allergic asthma, bronchial inflammation

Introduction

Zedoariae rhizoma, Curcuma zedaria R (Zingiberaceae), have been used as a Korean, Chinese and Japanese herbal medicine, which is listed in the Korean Pharmacopoeia II as aromatic stomachic, emmenagogus, or for the treatment of 'Ohyul extravasated blood' syndrome caused by blood stagnation. Furthermore, *Zedoariae rhizoma* also have been used as an important fragrance and spice in Asian countries. As chemical constituents of this plant, many sesquiterpenes, such as furanogermenone[1], germacrone[2,3], and (+)-germacrone 4,5-epoxide[4-12,17], have been isolated from *Zedoariae rhizoma*, and these sesquiterpenes have been reported to exhibit antihepatotoxic and anti-ulcer effects[1,4].

With respect to its bioactive constituents of natural medicines and medicinal foodstuffs[4-12], it has been reported that the sesquiterpene constituents from *Zedoariae rhizoma* exhibited potent vasorelaxant activity. In addition, absolute stereostructures of carabrane-type sesquiterpenes, curcumenone, 4S-dihydrocurcumenone, and curcarabranols A and B, were determined on the basis of physicochemical and chemical evidence[13]. Furthermore, it was also communicated

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on hepatoprotective activity of the 80% aqueous acetone extract and several known constituents from *Zedoariae rhizoma* and their plausible mechanisms of action[14].

Allergic asthma is a chronic inflammatory disease associated with a predominant Th2 response, immunoglobulin (Ig)E synthesis, airway infiltration by eosinophils, and bronchial hyperreactivity[15]. Allergic responses can be divided into two phases systemic response in the induction phase and allergic inflammation in the eliciting phase[16]. Naive clusters of differentiation CD4+ T cells initially stimulated with an allergen in the presence of interleukin (IL)-4 tend to develop into CD4+ T cells, which secrete IL-4, IL-5, IL-6, and IL-13 for IgE isotype switching. When the same allergen is inhaled, the allergen crosslinks performed IgE bound to high-affinity FcR on mast cells lining the bronchial mucosa, which consequently release stored mediators by granule exocytosis and synthesize leukotriens and cytokines. The allergic reaction is further promoted by the recruitment of Th2 cells, eosinophils and basophils in the airway. Th1 cells, into which naive CD4+T cells preferentially differentiate in the presence of IL-12, IL-15, IL-18, and IFN-γ, secrete IL-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α not only for induction of cell-mediated immunity but also for inhibition of Th2 responses[17]. Therefore, cytokines involved in Th1-based response are thought to regulate Th2-mediated allergic response. However, since these cytokines are known to be pleiotropic, the regulatory mechanism for Th differentiation by cytokines is still controversial. Allergic asthma is also the most common chronic disease in children. The prevalence of this disease is increasing rapidly, and its seriousness has become greater in recent years. There have been numerous descriptions of the clinical experiences of and prescriptions for asthma in traditional Korean medicine. However, the therapeutic mechanisms of these herbal medicines are unclear[18].

Many kinds of traditional herbal medicines have immunomodulating activities, e.g., B cell mitogenic activity[19], activation of macrophages[20], enhancement of natural killer (NK) activity[21] and action on hematopoietic stem cells. *Zedoariae rhizoma*, a traditional Korean medicine originating in China, is medical plant and used for treatment of symptoms such as weakness caused by fatigue and weakness after illness. In a preliminary study, an animal model of asthma was used to investigate the therapeutic effect of Korean berbs, a traditional Korean medicine used to treat bronchial asthma and allergic rhinitis for centuries. The results showed that *Zedoariae rhizoma* relieved the early and late asthmatic reactions, and reduced the local infiltration of inflammatory cells in the bronchoalveolar lavage fluid (BALF) of ovalbumin-sensitized guinea pigs. *Zedoariae rhizoma* has immunopharmacological activities such as augmentation of NK cell activity and suppression of IgE production in mice. Our previous study showed that *Zedoariae rhizoma* suppressed KLH-DNP-specific IgE response after primary immunization and that this effect was due to inhibition of the development of IL-4-producing CD4+T cells. These findings suggest that *Zedoariae rhizoma* inhibits allergic responses that are regulated by Th2 cells.

In this study, we examined the effect of oral administration of *Zedoariae rhizoma* on allergic responses using a murine model of asthma. We plan to use our established murine model of asthma to study the immunomodulatory effect of *Zedoariae rhizoma* on allergen-induced bronchial inflammation by analyzing the cellular distributions and T-cell subsets in the BALF of sensitized mice.

Material and methods

1. Reagents

Lyophilized house-dust mite (Dermatophagoides pteronyssinus [Der p]) was purchased from Allergon (Engelholm, Sweden). The crude mite preparation was extracted with ether. After dialysis with deionized water, the mite extract was lyophilized and stored at -20℃ before use. Monoclonal antibodies used for fluorescence-activated cell-scanning (FACScan) staining of CD4 (clone H129.19), CD8 (clone 53-6.7), and anti-CD3 (clone 145-2C11) were purchased from Pharmingen (San Diego, CA, USA). Mouse IgG1 and IgG2a conjugated with FITC or PE were purchased from Coulter Immunology (Hialeah, FL, USA) and used to determine the borderline between stained and unstained cells in the analysis of flow cytometry.

2. Mice

Female BALB/c mice were obtained from KCTC and used at 7-9 weeks of age.

3. Preparation of extract of Korean herbs

Dried *Zedoariae rhizoma* (3.0 kg, cultivated in Yongchun, Korea and purchased from Kyungju Market Co., Kyungju) were finely minced and extracted. The *Zedoariae rhizoma* was dissolved in distilled water for administration to mice. As for in vitro use, the water extract of *Zedoariae rhizoma* was isolated by dissolving *Zedoariae rhizoma* in distilled water, and then centrifuging the mixture at 7500 rpm for 30 min. After filtration, the aqueous extract was lyophilized and stored at -20℃. This extract of *Zedoariae rhizoma* was dissolved in pyrogen-free isotonic saline (Sigma Chemical, MO, USA) and

filtered through a 0.2 m filter (Microgen, Laguna Hills, CA, USA) before use. Spray-dried *Zedoariae rhizoma* was manufactured by a herb medicine hospital as the test drug. *Zedoariae rhizoma* was prepared as a hot water extract from medical plant(20 g). Extraction was carried out by boiling the mixture of herbs in 10 parts water at 95-100℃ for 1 h, and the extract was spray-dried in a hot air stream.

4. Mice, immunization, and allergen challenge (AC)

Specific pathogen-free, 6-8-week-old BALB/c mice from the Laboratory Animal Center, KRIBB, were used in this study. The mice were housed in microisolator cages (Laboratory Products, Maywood, NJ, USA) and provided with sterile food and water ad libitum. The colony was monitored regularly for the presence of murine pathogens. Results were consistently negative. All experimental animal care and treatment followed the guidelines set up by the National Health Council of the Republic of Korea. Groups of mice were subcutaneously injected at the base of the tail with 50 ㎕ of an emulsion containing 40 ㎍ of Der p in complete Freund's adjuvant (CFA, Difco, Detroit, MI, USA)[22]. Fourteen days later, the mice were lightly anesthetized with an intraperitoneal injection of 60 ㎎/㎏ of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago IL, USA). They received intratracheal (i.t.) instillation with 50 ㎕ of Der p (1 ㎎/㎖) as allergen challenge (AC). The animals were held in an upright position for 1 min to resume normal breathing.

5. Experimental groups for oral administration of Korean herbs

Mice sensitized with Der p were divided into three different groups for *Zedoariae rhizoma* administration. Group A was given 1 g of *Zedoariae rhizoma* extract per kilogram of body weight (g/kg) 1 h before Der p challenge; group B was given 1 g/kg *Zedoariae rhizoma* 24 h after AC; and group C was given 1 g/kg of *Zedoariae rhizoma* every other day six times, with the last treatment 48 h before AC. The control group was also sensitized with allergen but fed with PBS alone. Animals without sensitization, i.e., the naive group, or with sensitization but without *Zedoariae rhizoma* treatment (the control group), were also included in the experiment for purposes of comparison. There were six mice in each group for every experimental condition.

6. Bronchoalveolar lavage fluid (BALF)

The mice were killed with an overdose of sodium pentobarbital at various intervals after challenge. BALF was collected with two separate 1 ㎖ sterile endotoxin-free saline washes of lung via the trachea of each mouse. About 1.8 ㎖ of the washing solution was consistently recovered. BALF cells were washed once with HBSS (Life Technologies, Grand Island, NY, USA) containing 2% fetal calf serum by centrifugation at 200 g at 4℃. Red blood cells in BALF were removed in lysing solution containing 20 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA. After washing, the cells were resuspended in 1 ㎖ of HBSS. The recovered BALF was pooled, passed through a double layer of gauze to remove gross mucus, and then centrifuged. Total leucocytes in BALF was determined with a hemocytometer, and the results were expressed as cell number per milliliter. The aliquot was then diluted to a concentration of $1x10^5$ cells per milliliter, and 0.2 ㎖ cell suspension was spun down onto a glass slide at 1100 rpm for 2 min with a cytocentrifuge (Cytospin 2, Shandon Instruments, Sewickley, PA, USA). The slides were then dried and stained by the May-Giemsa method. More than 200 cells were identified with a photomicroscope. All samples were evaluated in a double-blind manner. The remaining BALF cells were resuspended in RPMI-1640 (GIBCO/BRL, Life Technologies, Inc, Gaithersburg, MD, USA) with 10% heat-inactivated fetal bovine serum (GIBCO/BRL), and incubated in plastic dishes for 60 min at 37℃ in 5% CO_2-humidified atmosphere to remove adherent macrophages that could interfere with accurate cell analysis. More than 90% of nonadherent cells collected for flow cytometric analysis were viable by the trypan blue exclusion test.

7. Flow cytometry analysis of lymphocytes in BALF

Specific binding of monoclonal antibody (mAb) was analyzed by direct immunofluorescence with a cytofluorograph (FACscan, Becton-Dickinson) by standard methods recommended by the Becton-Dickinson Monoclonal Center (Mountain View, CA, USA). Briefly, 50 ㎕ of cell suspension ($1.2x10^5$ cells) was incubated in the presence of saturating concentrations of fluoresein- or phycoerythrin-conjugated mAb in the dark on ice for 30 min. Erythrocytes were lysed by adding 3 ㎖ lysing solution (0.155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.3, for 3-5 min. Leucocytes were washed twice with PBS containing 1% BSA and 0.1% sodium azide. Cytofluorometric analysis was performed with scatter gates set on the lymphocyte fraction by forward and side scatter (SCC) and PE fluorescence FL2 with laser excitation at 488 nm. The number of immunofluorescence-positive cells was determined out of 10,000 cells analyzed. Specific binding of mAb was controlled by subtraction of isotype-matched rat immunoglobulin. A computer system (Consort 30, Becton Dickinson) was used for data acquisition and analysis. List mode data for 5000 to 10,000

events were stored. A cell gate containing lymphocytes was established on the basis of forward and side light scatter. For determination of the borderline between stained and unstained cells, cells were also stained with mouse IgG1-conjugated FITC or PE. Percentages were calculated on the basis of the number of lymphocytes found in each quadrant.

8. Preparation of intrapulmonary lymphocytes

Mice were also killed by cervical dislocation. For each experiment, three to six mice were used, and the lung cells pooled for analysis, as described by Abraham et al.[23]. In brief, the chest of the mouse was opened with two longitudinal incisions parallel to the sternum, avoiding the mammary vessels. The lung vascular bed was flushed by injecting 3-5 ㎖ of chilled (4℃) BBS (balanced salt solution) without phenolphthalein into the right ventricle. The lungs were then excised, avoiding the peritracheal lymph nodes, and washed twice in BSS. The lungs were minced finely, and incubated in RPMI 1640 with 5% FCS, penicillin/streptomycin, 10 mM HEPES, 50 μM 2-ME, and 20 mM L-glutamine, containing 20 U/㎖ collagenase (C2139, Sigma Chemical Co., St Louis, MO, USA) and 1 ㎍/㎖ Dnase (type I, Sigma). A volume of 25 ㎖ was used for four to six sets of lungs. After incubation for 60 min at 37℃ on a rotary agitator (approximately 60 rpm), any remaining intact tissue was disrupted by passage through a 21-gauge needle. Tissue fragments and the majority of dead cells were removed by rapid infiltration through a glass-wool column, and the cells were collected by centrifugation. The cell pellet was suspended in 4 ㎖ of 40% Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 ㎖ of 80% Percoll. After centrifugation at 600 g for 20 min at 15℃, the cells at the interface were collected, washed twice in BSS, and counted. Viability as determined by trypan blue exclusion was consistently more than 98%. Cells to be used for the lymphocyte proliferation assay were plated in 24-well culture plates, and were allowed to adhere for 1 h at 3 7℃ in a moist, 5% CO2 incubator to remove macrophages. Nonadherent cells were collected and diluted into RPMI 1640, supplemented, as above, with HEPES, glutamine, penicillin/streptomycin, 2-ME, and 2% FCS.

9. Determination of allergen- and mitogen-induced lymphocyte proliferation by 3H-labeled thymidine incorporation

Nonadherent intrapulmonary lymphocytes (5x104/㎖) were incubated in 0.1 ㎖ of the mentioned medium for assessment of proliferation by the incorporation of 3H-labeled thymidine (20 μCi/mmol, New England Nuclear, Boston, MA, USA). For determination of the inhibitory effect of *Zedoariae rhizoma* on allergen- or mitogen-induced lymphocyte proliferation, the extract of *Zedoariae rhizoma* was first incubated with cells at various concentrations and incubation times, and then washed with medium five times to remove *Zedoariae rhizoma* before allergen or mitogen (PHA or LPS) was added to induce cell proliferation. Allergen or mitogen solution was diluted in the same medium, and an aliquot of 0.1 ㎖ was added to each well of a round-bottom, 96-well microtiter plate (Nunc, Denmark). Cells were incubated for 60 h at 37℃ and 5% CO_2 incubator. For these experiments, 0.5 μ Ci/mmol of 3H-labeled thymidine was added to each well, and incubation was continued for another 12 h. Cells were then harvested and repeatedly washed with a PhD cell harvester. Subsequently, incorporated thymidine was evaluated by measuring radioactivity in a liquid scintillation counter. All results are expressed as mean values in triplicate.

10. Immunization with OVA and treatment of *Zedoariae rhizoma*

Mice were intraperitoneally immunized with 100 g OVA absorbed on 100 ℓ of incomplete Freund's adjuvant (IFA) on days 0 and 7. The mice were inhalated with 1% OVA solution on days 14, 16, 18, and 20. In *Zedoariae rhizoma* treatment at the induction phase, the mice were orally administered 1000 ㎎/㎏ of *Zedoariae rhizoma* (suspended in phosphate-buffered saline (PBS)) or PBS on days 1-8 (Fig. 1). In the eliciting phase treatment, oral administration of *Zedoariae rhizoma* (1000 ㎎/㎏) or PBS was begun 6 days after the last immunization (Fig. 4). Sera were obtained at 2-week intervals after immunization and stored at -20℃ until analysis. Spleen cells and bronchoalveolar lavage fluid (BALF) were prepared 24 h after the last inhalation.

11. Measurement of OVA-specific IgE, IgG1, and IgG2a

Levels of OVA-specific IgE, IgG1, and IgG2a were determined by ELISA. Sample wells of an ELISA plate were coated with OVA overnight and then blocked with 1% BSA in borate-buffered saline (0.05 M borate, 0.15 M NaCl, pH 8.6, 100 l/well) at 37℃ for 30 min. Diluted samples (100 ℓ/well) were incubated for 90 min at room temperature (samples for IgE, IgG1 and IgG2a were diluted 1:100, 1:1000 and 1:5, respectively). The plates were washed with borate-buffered saline with 0.05% Tween 20 and incubated with peroxidase-conjugated anti-mouse IgE, IgG1or IgG2a (Nordic Immunology) for 90 min at room temperature. After further washing, plates were incubated for 20 min at room temperature with 100-well of o-phenylendiamine solution (1 g/ ㎖ with 3% H_2O_2), and OD was read at 492 nm.

12. Analysis of cytokines in BALF

To obtain BALF, mice were anesthetized, a tracheal cannula was inserted in each mouse via a midcervical incision, and the airway of each mouse was lavaged three times with 1 ml of PBS. BALF was immediately centrifuged (10 min, 4℃, 160×g), and the supernatant was rapidly frozen. Commercial ELISA kits were used to measure the levels of IL-4 and IFN-γ (Genzyme Diagnostics) and IL-5 (Genzyme TECHNE, AN'ALYZA TM, Immunoassay system) in the BALF.

13. Staining of BALF cells

BALF cells were stained by the Pappenheim staining method using modified May-Gruenwald's solution (Merck) and Giemsa's stain solution (Katayama Chemical), and the cells were identified as eosinophils, neutrophils, macrophages and lymphocytes by standard morphology. At least 100 cells were counted, and the absolute number of each cell type was calculated.

14. Measurement of cytokine production by spleen cells

Spleen cells were incubated on a nylon wool column at 37℃ in 5% CO_2 for 60 min. T cells (5×10^5) and MMC-treated naive splenocytes (5×10^5) were cultured in 96-well cell culture plates (Falcon, Becton Dickinson) with 200 g OVA. After 48 h of culture, the cultured supernatants were collected and the amounts of secreted IL-4 and IFN- in the supernatants were determined by ELISA. For cellular proliferation, the cultures were pulsed with 1 μCi/well of [^{3}H]TdR for an additional 6 h after 48 h of culture. [^{3}H]TdR incorporation was determined by liquid scintillation counting.

15. Statistical analysis

Results were all shown as mean±SEM and analyzed either by Student's t-test or by analysis of variance. Comparison of group means was done with the Scheffes F test when all possible pairwise comparisons were made. Differences of $P<0.05$ were judged to be significant. All experiments were repeated at least twice. The data of study (with *Zedoariae rhizoma* in different protocols) and control groups were analyzed and compared by one-way ANOVA.

Results

1. Effect of korean herbs administration on cellular distribution in BALF of naive BALB/c mice

Previously, we have tested the oral single dose toxicity of *Zedoariae rhizoma* on male and female Spraque-Dawley rats. The LD50 of *Zedoariae rhizoma* was estimated to be greater than 8 g/kg. No observable abnormal clinical signs were attributable to the *Zedoariae rhizoma* dosing, and there was neither loss of body weight nor grossly abnormal findings at autopsy. The changes of cellular distribution in BALF of naive mice at 24 h after treatment with various doses of *Zedoariae rhizoma* extract are shown in Table 1. There was a marked increase in the percentage of lymphocytes and decrease of macrophages in the BALF after administration of 1g/kg and 2 g/kg of *Zedoariae rhizoma*, respectively. The total cell counts of BALF were also significantly increased in mice treated with 1 g/kg of *Zedoariae rhizoma* as compared to those of untreated (0 g/kg) mice. We have found that the increased percentage of lymphocytes in the BALF of *Zedoariae rhizoma*-treated naive mice was due to the increased subset of $CD3^+/CD8^+$ (58±6% vs 20±9%) and $CD4^-/CD8^-$ double-negative T cells (12±3% vs 3±1%) as compared to those of untreated mice. The change of cellular distribution in the BALF of naive mice treated by *Zedoariae rhizoma* was dose- and time-dependent, as shown in Table 1 and Fig. 1. These cellular changes in BALF were most significant at 72 h when the mice were treated with 1 g/kg of *Zedoariae rhizoma*, and gradually subsided to basal levels at day 7 after feeding(Fig. 1).

Table 1. Changes of total cell counts and cellular distributions in BALF of naive mice 24 h after oral administration of *Zedoariae rhizoma*.

Oral administration (g/kg)	Total cell counts	Macrophages	Lymphocytes	Neutrophils	Eosinphils
0	32.4	22.3	1.3	0.1	0
0.5	23	14.7	7.4	0.6	0
1	71.3	34.7	29.5	1.1	0
2	34.6	13.5	11.4	0.8	0

*P<0.05, as compared to untreated (0 g/kg) mice.

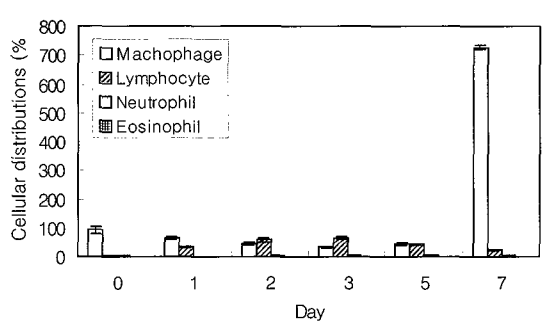


Fig. 1. Change of cellular distributions in BALF of naive BALB/c mice after oral administration of 1 g/kg of *Zedoariae rhizoma* extract.

2. The prophylactic or therapeutic effect of *Zedoariae rhizoma* on allergen-induced airway inflammation of sensitized mice

The changes of total cell counts and cellular distributions in BALF of groups of sensitized mice 72 h after allergen challenge are shown in Table 2. Intratracheal (i.t.) challenge of 50 μℓ Der p (1 ㎎/㎖) of sensitized mice without *Zedoariae*

rhizoma treatment (controls) induced a marked increase in the number of infiltrated cells (Fig. 2a) and percentage of eosinophils in BALF (Fig. 2b) by 24 h after AC. Levels of the infiltrated cells peaked at day 3 and then gradually returned to baseline around day 7 after AC. The percentage change of eosinophils had the same pattern as total cellular infiltration in BALF that peaked at day 3, except that it remained high even at day 7 after AC. For evaluation of the prophylactic effect of *Zedoariae rhizoma* on allergen-induced airway inflammation, sensitized mice were given *Zedoariae rhizoma*(1 g/kg) 1 h before AC (group A), and the number of BALF cells and percentage of eosinophils were significantly lower than the PBS group at days 2, 3, and 7 after AC, respectively. Moreover, the percentage of lymphocytes and neutrophils (Table 2) also showed significant differences between group A and the control group (65.3% vs 28.4% in lymphocytes, and 23.4% vs 56.2% in neutrophils). There were also significantly lower numbers of infiltrated cells and percentage of eosinophils in the BALF at days 3 and 7 after AC of the sensitized mice that received six oral treatments of *Zedoariae rhizoma* before AC (group C) as compared to those of control mice. Regarding the therapeutic effect of *Zedoariae rhizoma*, there was no significant change in total cell infiltration and the percentage of eosinophils in the BALF of sensitized mice, when *Zedoariae rhizoma* was administered 24 h after AC (group B), as compared to those in controls.

Table 2. Changes of total cell counts and cellular distributions in BALF of groups of sensitized mice 72 h after allergen challenge

Groups	Total cells	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
Naive mice	44	38	3	0.5	0
Control	120	42.5	44.5	19.3	17.1
Group A	80	14.3	46.7	4.3	2.9
Group B	165	12.3	50.5	17.2	26.7
Group C	72	13.2	38.4	3.1	3.3

*P<0.05, as compared with PBS-treated sensitized (controls) mice.

3. Flow cytometry analysis of T-cell population in the BALF of sensitized mice

The effect of *Zedoariae rhizoma* on the percentage change of T-cell subsets was determined by flow cytometry with immunofluorescence of monoclonal antibodies to direct staining of CD3, CD4, and CD8 molecular on infiltrated lymphocytes in the BALF of sensitized mice (Fig. 3). There was significant decrease in the percentage of CD3+/CD4+ lymphocytes in groups A and C mice as compared to those of control mice, respectively. Moreover, the percentage of double-negative (CD4-/CD8-) T cells in the BALF of sensitized mice was also increased when mice were treated with single as well as multiple doses of *Zedoariae rhizoma* before AC. The percentage of CD3+/CD8+ lymphocytes was increased in groups A and C, but not to a statistically significant extent as compared to controls. The change of T-cell subsets in the BALF of group B mice was not significantly different from those of controls.

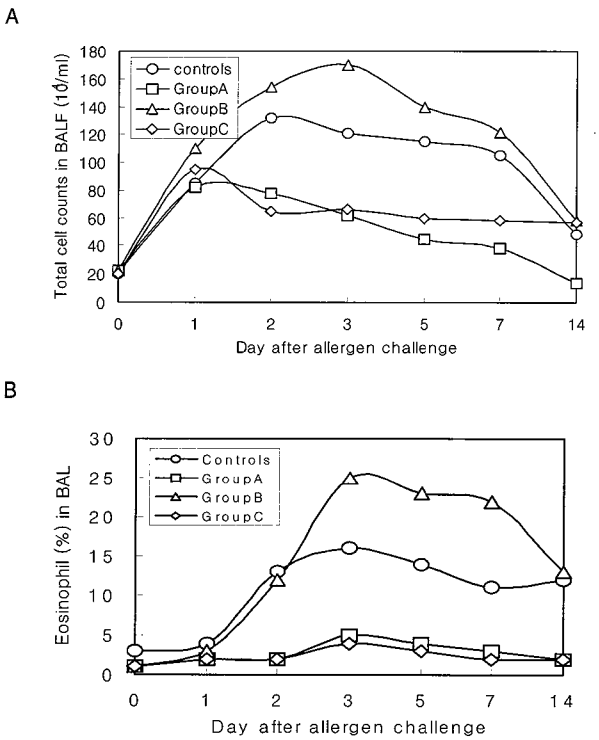


Fig. 2. Changes of total cell counts (a) and percentage of eosinophils (b) in BALF of sensitized mice after allergen challenge with different treatments of *Zedoariae rhizoma* or PBS. *P<0.05, as compared with PBS treated (controls) sensitized mice.

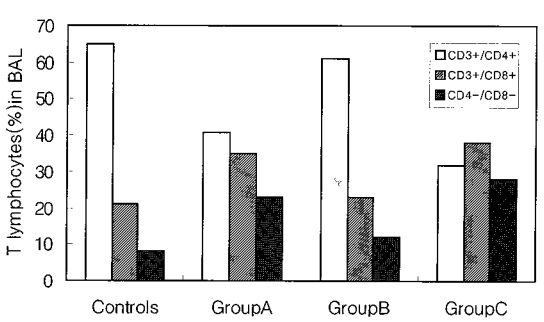


Fig. 3. Effect of Zedoariae rhizoma on T-cell subsets in BALF of sensitized mice 72 h after AC. *P<0.05, as compared with PBS group.

4. Effect of *Zedoariae rhizoma* on the allergen- or mitogen-induced intrapulmonary lymphocyte proliferation

We have also isolated intrapulmonary lymphocytes from sensitized mice at day 3 after AC to assay the inhibitory effect of *Zedoariae rhizoma* on allergen- or mitogen-induced lymphocyte proliferation. The results in Fig. 4 show that there was dose-dependent inhibition of *Zedoariae rhizoma* on PHA-, LPS- or Der p-allergen-stimulated lymphocyte proliferation in

the sensitized mice. *Zedoariae rhizoma* had a significant suppressive effect on Der p-stimulated lymphocyte proliferation at the concentrations of 10 ㎍/㎖, as compared to Der p-stimulated cells alone ($P<0.05$). Similarly, PHA- or LPS-stimulated lymphocyte proliferation was also suppressed in the presence of *Zedoariae rhizoma* extract, but was not statistically significantly different from that of mitogen-stimulated lymphocytes without *Zedoariae rhizoma*.

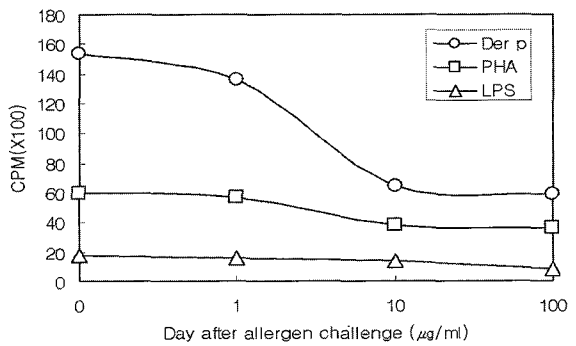
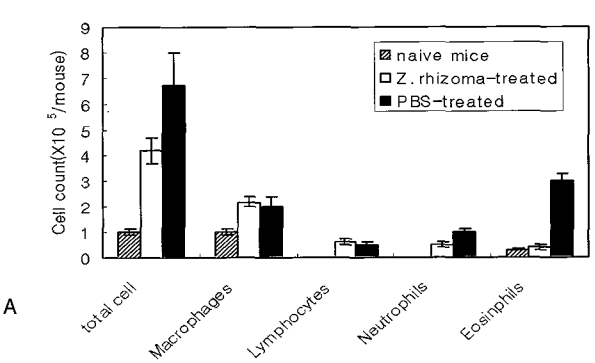


Fig. 4. Effect of *Zedoariae rhizoma* on allergen- or mitogen-induced intrapulmonary lymphoproliferation. *P<0.05, as compared with mitogen-stimulated lymphocytes without korean herbs.

5. Impaired allergic inflammation in the lungs of mice treated with *Zedoariae rhizoma* in the induction phase

BALB/c mice were intraperitoneally sensitized with OVA/IFA on days 0 and 7. In the experiment on the effect of administration in the induction phase of allergic responses, mice were orally administrated with *Zedoariae rhizoma* or PBS every day from days 1 to 8 after the first immunization with OVA/IFA. The mice were then exposed to aerosolized OVA every second day from days 14 to 20. The BALF was recovered 24 h after the last inhalation. The numbers of total cells and eosinophils in the BALF were markedly increased in OVA-sensitized mice after OVA inhalation (data not shown). As shown in Fig. 5A, the numbers of total cells and eosinophils in OVA-sensitized mice treated with *Zedoariae rhizoma* in the induction phase were significantly lower than those in mice treated with PBS ($p<0.05$).



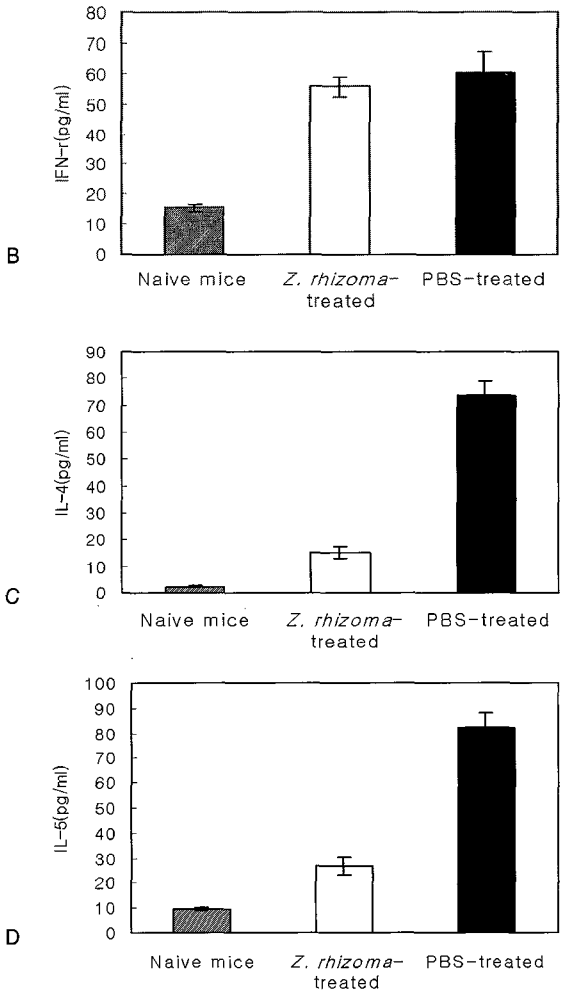


Fig. 5. Effect of in vivo administration of *Zedoariae rhizoma* in the induction phase on the cell composition and cytokine production in BALF after inhalation of OVA. All mice were immunized with OVA/IFA on days 0 and 7. The mice were then exposed daily to aerolized OVA for 30 min on days 14, 16, 18, 20. The mice were orally administrated with Zedoariae rhizoma or PBS from days 1 to 8. (A) BALF cell composition of eosinophils, macrophages, lymphocytes and neutrophils in naive mice (▨), *Zedoariae rhizoma*-treated (□) and PBS-treated (■) mice after inhalation of OVA. (B) BALF cytokine IFN-γ, (C) BALF cytokine IL-4 and (D) BALF cytokine IL-5 levels in naive mice (▨), OVA-sensitized Zedoariae rhizoma-treated (□) and PBS-treated (■) mice after inhalation of OVA. Each bar represents data from at least three independent experiments using 6- to 8-week-old mice. The values are expressed as means±S.D. *, $p<0.05$.

We examined the cytokine production in the BALF after a challenge with OVA inhalation. As shown in Fig. 5B, C and D, the levels of IL-4 and IL-5 were significantly decreased in the BALF of *Zedoariae rhizoma*-treated mice compared with those in PBS-treated mice. There was no difference between the levels of IFN-γ in the *Zedoariae rhizoma*-treated and PBS-treated mice. These results suggest that OVA-induced airway inflammation is severely reduced in mice administered *Zedoariae rhizoma* in the induction phase.

6. Impaired Th2 responses in mice treated with *Zedoariae rhizoma* in the induction phase

To determine which type of T cell responses preferentially developed in the mice treated with *Zedoariae rhizoma* in the induction phase by OVA sensitization, we assessed OVA-specific IgE/IgG2a production in the serum on days 0, 14 and 21 after the first immunization with OVA/IFA. The PBS-treated mice had a higher OVA-specific IgE level in the serum than did the *Zedoariae rhizoma*-treated mice after OVA sensitization (Fig. 6A,B,C). On the other hand, the level of OVA-specific IgG2a was significantly higher in the *Zedoariae rhizoma*-treated mice than in the non-treated mice sensitized and inhalated with OVA (Fig. 6A,B,C).

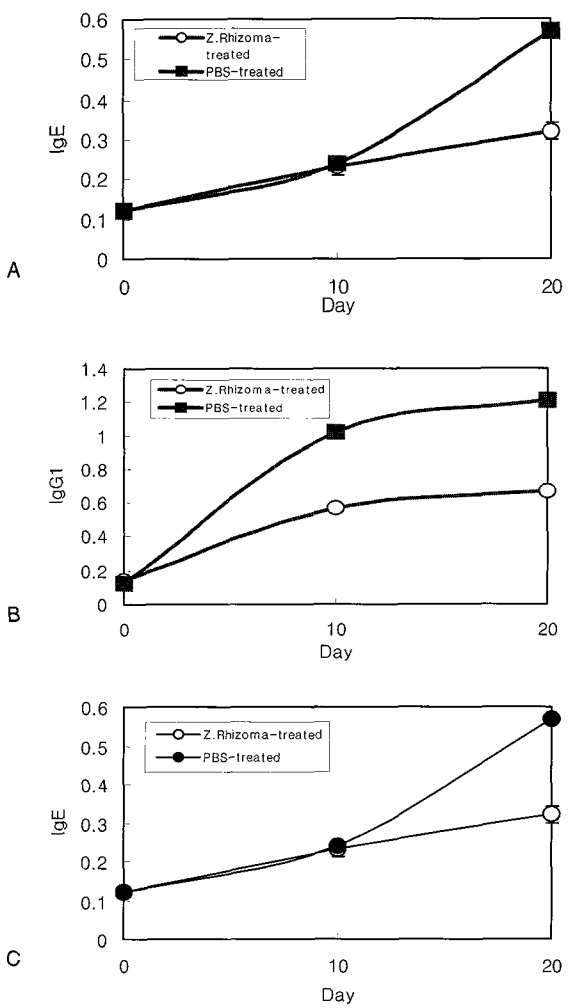


Fig. 6. Serum levels of OVA-specific Igs in Zedoariae rhizoma-treated or PBS-treated mice in the induction phase. Individual levels of OVA-specific Igs were determined by ELISA in Zedoariae rhizoma-treated or PBS-treated mice on days 0, 14, and 21. Data were obtained from three independent experiments and are expressed as the means of triplicate determinations±S.D. Per experimental group, three to four mice were used. *, $p<0.05$.

We separated T cells from the spleen of mice treated with *Zedoariae rhizoma* or PBS in the induction phase. As shown in Fig. 7A,B,C, $CD3^+$T cells of PBS-treated mice sensitized with OVA produced IL-4 in response to OVA, whereas the IL-4 production was significantly reduced in *Zedoariae rhizoma*-treated mice sensitized with OVA. The IFN-γ production in response to OVA was increased in the culture supernatant of spleen T cells from *Zedoariae rhizoma*-treated mice sensitized with OVA. Thus, Th2 responses were suppressed by oral administration with *Zedoariae rhizoma* in the induction phase but Th1 responses were augmented in these mice.

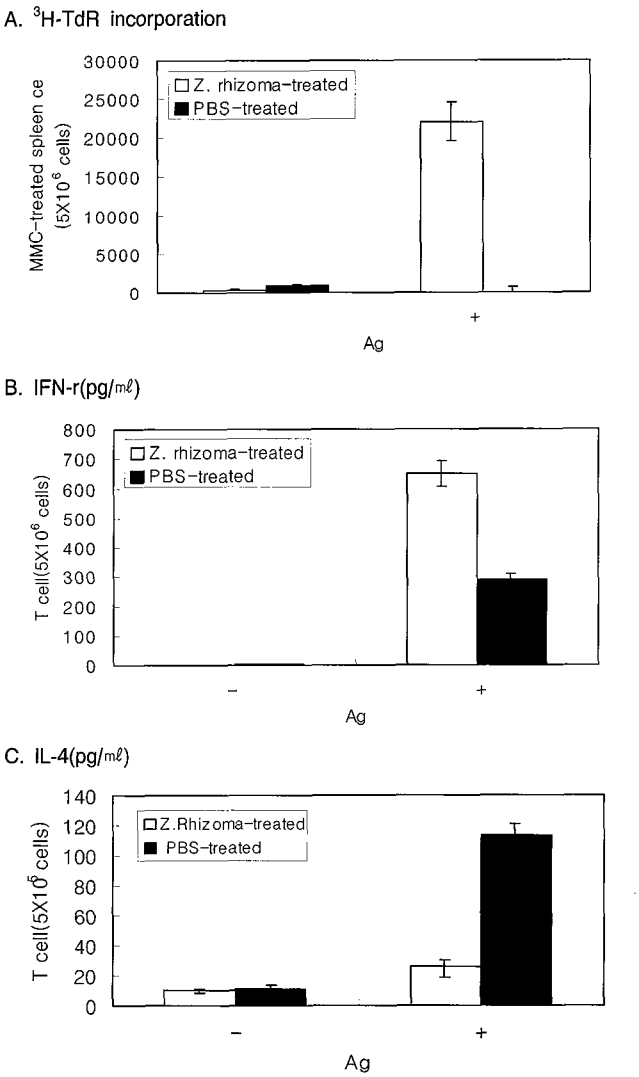


Fig. 7. Cytokine production by spleen T cells from Zedoariae rhizoma-treated or PBS-treated mice at the induction phase. The enriched T cells (5×10^5 cells) from the spleen of Zedoariae rhizoma-treated (□) or PBS-treated (■) mice sensitized with OVA were cultured with OVA in the presence of MMC-treated spleen cells (5×10^5 cells) for 48 h at 37℃. All mice were immunized with OVA/IFA. The proliferation activities of spleen T cells was assessed by incorporation of [^{3}H]TdR. IL-4 and IFN-γ production by the T cells was assessed by ELISA. The data are representative of four independent experiments using pooled cells from three mice and are shown as mean of triplicate determinations±S.D. *, $p<0.05$; **, $p<0.001$.

7. Increment of allergic inflammation in the lungs of mice treated with *Zedoariae rhizoma* in the eliciting phase

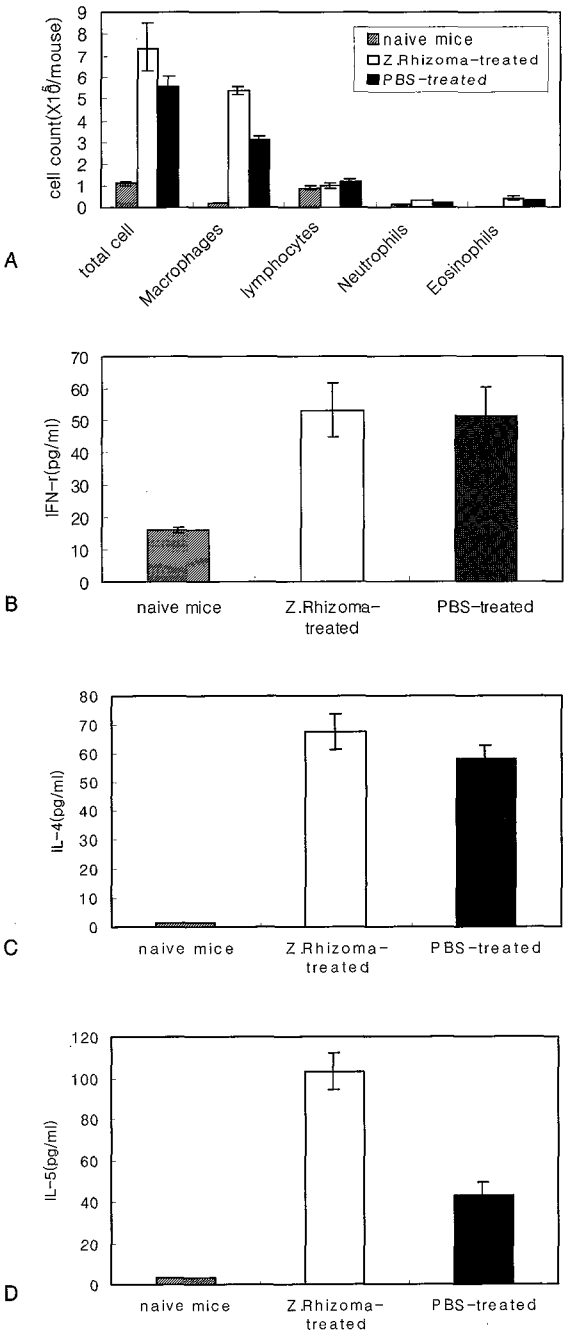
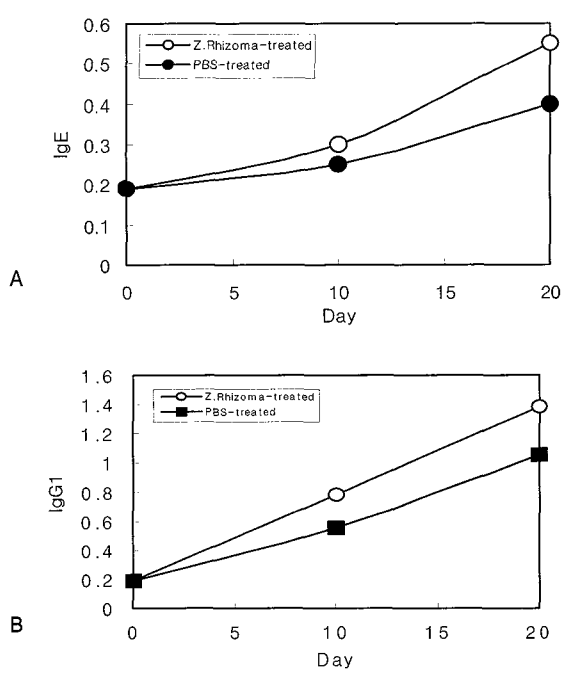


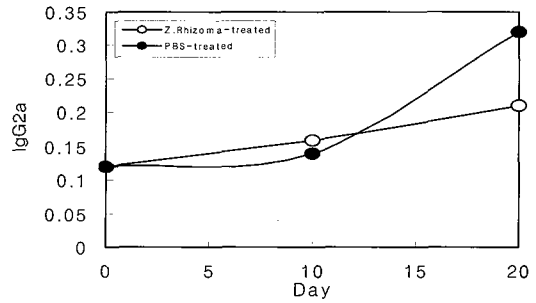
Fig. 8. Effect of in vivo administration of *Zedoariae rhizoma* in the eliciting phase on the cell composition and cytokine production in BALF after inhalation of OVA. All mice were immunized with OVA/IFA on days 0 and 7. The mice were then exposed daily to aerolized OVA for 30 min on days 14, 16, 18, 20. The mice were orally administrated with Zedoariae rhizoma or PBS every day from days 13 to 20. (A) BALF cell composition of eosinophils, macrophages, lymphocytes and neutrophils in naive mice (▨), Zedoariae rhizoma-treated (□) and PBS-treated (■) mice after inhalation of OVA. (B) BALF cytokine IFN-γ levels in naive mice (▨), OVA-sensitized Zedoariae rhizoma-treated (□) and PBS-treated (■) mice after inhalation of OVA. C) BALF cytokine IL-4 levels in naive mice (▨), OVA-sensitized Zedoariae rhizoma-treated (□) and PBS-treated (■) mice after inhalation of OVA. D) BALF cytokine IL-5 levels in naive mice (▨), OVA-sensitized Zedoariae rhizoma-treated (□) and PBS-treated (■) mice after inhalation of OVA.Each bar represents data from at least three independent experiments using 6- to 8-week-old mice. The values are expressed as means±S.D. *, $p<0.05$.

We next examined the effect of *Zedoariae rhizoma* treatment in the eliciting phase of allergic responses in vivo. Mice were orally administered *Zedoariae rhizoma* or PBS once every day from days 13 to 20 after the first immunization with OVA/IFA. As shown in Fig. 8A, the numbers of total cells and eosinophils in the BALF of OVA-sensitized mice treated with *Zedoariae rhizoma* in the eliciting phase after OVA inhalation were slightly higher than those in mice treated with PBS. The level of IL-5 was increased in the BALF of OVA-sensitized mice treated with *Zedoariae rhizoma* after OVA inhalation (Fig. 8B,C,D). There were no differences between the levels of IL-4 or between the levels of IFN- in the *Zedoariae rhizoma*-treated and non-treated mice(Fig. 4B,C.D).

8. Increment of Th2 responses in mice treated with *Zedoariae rhizoma* in the eliciting phase

We assessed OVA-specific IgE/IgG2a production in the serum on days 0, 14 and 21. As shown in Fig. 9, the mice treated with *Zedoariae rhizoma* in the eliciting phase had a higher OVA-specific IgE level in the serum than did PBS-treated mice after OVA-sensitization ($p<0.05$). On the other hand, the level of OVA-specific IgG2a was significantly lower in the *Zedoariae rhizoma*-treated mice than that in the PBS-treated mice. We next examined the cytokine production in response to OVA by splenic T cells from mice treated with *Zedoariae rhizoma* in the eliciting phase. As shown in Fig. 10, the level of IL-4 was slightly increased in the culture supernatant of spleen T cells from mice treated with *Zedoariae rhizoma* at the eliciting phase. However, there was no difference between the amounts of IFN-γ produced in response to OVA in the mice treated with PBS and those treated with *Zedoariae rhizoma* in the eliciting phase.

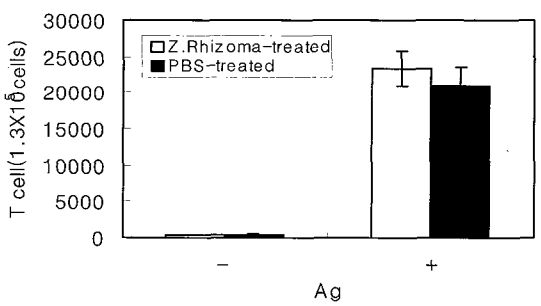




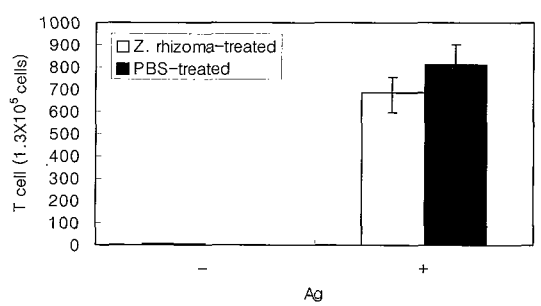
C

Fig. 9. Serum levels of OVA-specific Igs in Zedoariae rhizoma-treated or PBS-treated mice in the eliciting phase. Individual levels of OVA-specific Igs were determined by ELISA in Zedoariae rhizoma-treated or PBS-treated mice on days 0, 14, and 21. Data were obtained from three independent experiments and are expressed as the means of triplicate determinations±S.D. Per experimental group, three to four mice were used. *, $p<0.05$; **, $p<0.001$.

A. ^{3}H TdR incorporation



B. IFN-r(pg/ml)



C. IL-4(pg/ml)

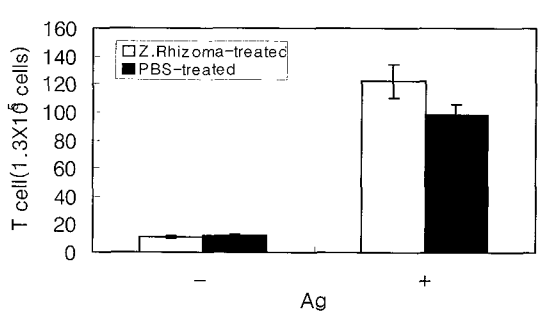


Fig. 10. Cytokine production by spleen T cells from Zedoariae rhizoma-treated or PBS-treated mice in the eliciting phase. The enriched T cells (1.3×10^5 cells) from the spleen of *Zedoariae rhizoma*-treated (□) or PBS-treated (■) mice sensitized with OVA were cultured with OVA in the presence of MMC-treated spleen cells (1.3×10^5 cells) for 48 h at 37℃. All mice were immunized with OVA/IFA. The proliferation activities of spleen T cells was assessed by incorporation of [^{3}H]TdR. IL-4 and IFN- production by the T cells was assessed by ELISA. The data are representative of four independent experiments using pooled cells from three mice and are shown as mean of triplicate determinations±S.D. *, $p<0.05$; **, $p<0.001$.

Discussion and Conclusion

It was recently demonstrated that allergens of house-dust mites may induce eosinophilia in sensitized mice[22,24]. Such eosinophilia was $CD4^+$ T-cell dependent and associated with a series of inflammatory and immunologic events, including the development of early-type hypersensitivity, tumor necrosis factor (TNF)-α production, VCAM-1 expression, and tracheal hyperreactivity. In the present study, a murine model of asthma, prepared and characterized as described, was designed to evaluate the prophylactic and/or therapeutic effect of the Chinese herbal medicine on allergen-induced lymphocyte proliferation and airway inflammation in dust-mite-allergen (Der p)-sensitized BALB/c mice. We have shown that there was significant decrease in the amount of cellular infiltration and percentage of eosinophils in the BALF of sensitized mice that had been treated with single and multiple doses of *Zedoariae rhizoma* before AC. Moreover, the percentage of lymphocytes in the BALF was significantly increased in group A and group C as compared to that of the control group. Flow cytometry analysis has shown that this increase of lymphocyte percentage in BALF was mainly due to increase in the percentage of the $CD8^+$ T-cell subset and, to a minor degree, to double-negative ($CD3^+/CD4^-/CD8^-$) T cells.

T cells of a Th2-like phenotype[25,26] are thought to play an important role in orchestrating the asthmatic inflammatory response. When atopic asthmatics are exposed to allergen, it has been found that $CD4^+$ T cells are depleted in the circulation and sequestered in the lung[26]. Moreover, an elevation in double-negative T cells was observed in subjects who develop isolated early asthmatic response, but not late-phase response. It is proposed that the function of $CD4^-/CD8^-$ cells is to downregulate or suppress the late inflammatory response[27]. Therefore, the immunomodulatory role of *Zedoariae rhizoma* in the allergen-sensitized mice may lie in regulation of T-cell activation. Further studies are needed to verify the effect of *Zedoariae rhizoma* in changing T-cell subsets during allergic inflammation.

Preliminary study showed that administration to mice of 8 g/kg of *Zedoariae rhizoma* daily would induce a high energy state, and cause no harm to the liver, as shown by autopsy. In order to determine whether there was any therapeutic and/or long-term prophylactic effect of *Zedoariae rhizoma* on allergen-induced airway inflammation, we used three different protocols in administration of 1 g/kg of *Zedoariae rhizoma* extracts to sensitized mice 1 h before AC (group A), 24 h after AC (group B), and every other day six times before AC (group C). We found that group A and group C showed the same prophylactic effect of reduced cellular infiltration and

decreased eosinophil percentage in the BALF during allergen-induced bronchial inflammation. However, at 24 h after AC and at the start of the late phase of allergic inflammation, the administration of *Zedoariae rhizoma* did not significantly suppress the cellular infiltration in the BALF of group B sensitized mice as compared to those of the control group. Moreover, the percentage of eosinophils in the BALF of group B mice was markedly increased when they were treated with *Zedoariae rhizoma* after AC. We are still unable to explain the different response of these two protocols in sensitized mice. It may be due to the allergen-induced bronchial inflammatory process in the sensitized mice, and the fact that the *Zedoariae rhizoma* treatment did not only lose its suppressive effect, but it also enhanced the already activated CD4 T-cell function of recruiting more eosinophils and worsening the inflammation of the lung. Of course, this conception of the *Zedoariae rhizoma* pharmacologic function must be confirmed by further study.

In a preliminary study, we have also found that *Zedoariae rhizoma* inhibited LTB4 production, but had no effect on histamine release by peripheral blood mononuclear cells from Der p-sensitized asthmatic children (unpublished data). Therefore, the different responses between group A (prophylactic) and group B (therapeutic) may be due to the anti-inflammatory effect of *Zedoariae rhizoma* on the different phases of allergen-induced airway inflammation.

In this study, we assessed the efficacy of *Zedoariae rhizoma* treatment in the induction phase or the eliciting phase in a murine model of asthma. When oral administration of *Zedoariae rhizoma* was begun immediately after OVA sensitization, Th2 responses were reduced and allergic inflammation in the lung caused by OVA inhalation was significantly suppressed. In contrast, oral administration of *Zedoariae rhizoma* in the eliciting phase, well after the Th2 response had developed, aggravated the allergic inflammation after OVA inhalation. *Zedoariae rhizoma* may be applicable for prophylactic use to prevent allergy response but must be used carefully for patients with apparent allergic inflammation.

Atopic asthma is a complex phenomenon driven predominantly by Th2-type cells[16,28]. Asthma is characterized by the overproduction of Th2 cytokines, which initiate and sustain the allergic asthmatic inflammatory responses by enhancing the production of IgE and the growth, differentiation, and recruitment of mast cells, basophils, and eosinophils. The development of atopic asthma is related to immediate hypersensitivity. Thus, besides bronchodilators, anti-allergic agents are widely used for the treatment of asthma. Recently, it has been reported that treatment with various traditional medicines had a beneficial effect for patients with asthma[29,30]. we reported that oral administration of *Zedoariae rhizoma* decreased the serum level of KLH-DNP-specific IgE. In the present study, we found that oral administration of *Zedoariae rhizoma* in the induction phase inhibited allergic inflammatory responses in a murine model of asthma. Thus, studies on these Korean Traditinal Medicines may lead to the development of agents that can prevent asthma.

Impaired airway inflammation is mostly due to insufficient induction of Th2 responses for IgE production in the periphery. Th2 response is inhibited by IFN-γ-producing Th1 cells. Clinical studies have demonstrated that reduced IFN-γ secretion in neonates is associated with the subsequent development of atopy[31,32]. Furthermore, a predisposition toward the overproduction of Th1 cytokines may protect against atopy, because patients with multiple sclerosis[33], rheumatoid arthritis[34] or tuberculosis[35], conditions associated with increased production of Th1 cytokines, have a reduced predisposition toward the development of atopy. In the present study, we found that oral administration of *Zedoariae rhizoma* in the induction phase preferentially induced Th1 cells producing IFN-γ. At present, the mechanism for induction of Th1 response by *Zedoariae rhizoma* administration remains unknown. Macrophage/dendritic cell-derived cytokines such as IL-12, IL-15, and IL-18 are at least partly responsible for early IFN-production from NK and T cells and consequently Th1 cell differentiation. *Zedoariae rhizoma* may stimulate macrophages/dendritic cells to produce these cytokines, resulting in predominant Th1 responses. Alternatively, *Zedoariae rhizoma* may contain ligands for NK and T cells and directly stimulate produced IFN-γ. Thus, the results of the present study suggest that methods to enhance IFN-γ production might be clinically useful in the prophylaxis of allergic asthma.

Surprisingly, the number of eosinophils and the level of IL-5 in BALF were increased in the mice treated with *Zedoariae rhizoma* in the eliciting phase after OVA inhalation. Th2 responses were also increased in these mice. These observations clearly indicate that the administration of *Zedoariae rhizoma* in the eliciting phase promotes Th2-type responses in the lung as an effector site. *Zedoariae rhizoma* may exacerbate rather than suppress Th2-type responses when administered during ongoing Th2-type responses. Recently, Hansen et al.[36] reported that antigen-specific Th1 cells cannot prevent Th2-mediated allergic disease but, rather, cause lung pathology. More recently, IL-15 and IL-18 have been reported to show pleiotropic effects on Th2 responses. Although IL-18 preferentially induces Th1 response in the presence of IL-12, IL-18 itself can preferentially induce Th2 response and IgE

production[37]. Similarly, IL-15 preferentially induces Th1/Tc1 responses in the presence of IL-12[38], but it can directly stimulate mast cells to produce IL-4[39]. Therefore, it is possible that such cytokines induced by *Zedoariae rhizoma* administration may affect allergic inflammation once Th2 response had been established. Alternatively, *Zedoariae rhizoma*-induced IFN-γ production may provoke pathological changes in allergic inflammation at the eliciting phase. From the therapeutic aspect, *Zedoariae rhizoma* should be used carefully for Th2-dominated allergic inflammation.

In summary, we found that water extract of *Zedoariae rhizoma* administered to allergen-sensitized mice 1 h before allergen challenge and every other day six times before AC suppressed airway inflammation by decreasing the amount of cellular infiltration and percentage of eosinophils in the BALF, and downregulating the allergen- or mitogen-induced intrapulmonary lymphocyte response. This immunomodulatory effect of *Zedoariae rhizoma* may be exerted through regulation of T-cell subsets by elevation or activation of the CD8+ and double-negative T-cell population. However, the administration of *Zedoariae rhizoma* to sensitized mice 24 h after AC did not have the same inhibitory effect on the airway inflammation as *Zedoariae rhizoma* given before AC. The mechanism of these two different responses of *Zedoariae rhizoma* remains unclear. In addition, oral administration of *Zedoariae rhizoma* reduced Th2 responses in the induction phase of allergic responses via preferential induction of Th1 responses, resulting in the inhibition of allergic inflammation in a murine model of asthma. On the other hand, *Zedoariae rhizoma* aggravated the allergic inflammation when administrated at the eliciting phase. These results thus offer a different approach using *Zedoariae rhizoma* for the treatment of allergic disorders. Further studies are needed to determine the precise role of this herbal medicine in the treatment of asthma.

Acknowledgment

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