



Liriope tuber inhibit OVA-induced airway inflammation and bronchial hyperresponsiveness in murine model of asthma

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Abstract

Liriope platyphylla is one of the well-known herb used in oriental medicine for treatment asthma and bronchial and lung inflammation. Anti-asthmatic effects of *Liriope platyphylla* in the development of OVA-induced airway inflammation and murine asthma model have not been fully investigated in vivo.

Asthma is a chronic inflammatory disease of the mucosa and is associated with excess production of Th2 cytokines and eosinophil accumulation in lung. To clarify the anti-inflammatory and anti-asthmatic effects of *Liriope platyphylla*, we examined the influence of *liriope tuber* (LRT) on the development of pulmonary eosinophilic inflammation in murine model of asthma.

Our results have shown that LRT were demonstrated on the accumulation of eosinophils into airways, with reduction of eosinophil, total lung leukocytes numbers by reduction IL-5, IL-13, IL-4 and IgE levels in the BALF and serum. Moreover, LRT decreased eosinophil CCR3 expression and CD11b expression in lung cells. These results indicate that LRT has a deep inhibitory effects on airway inflammation and hyperresponsiveness in murine model of asthma and play an crucial role as a immunomodulator which possess anti-inflammatory and anti-asthmatic property by modulating the relationship between Th1/Th2 cytokine imbalance.

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1. Introduction

Bronchial asthma is a chronic inflammatory disorder of the airways characterized by various airway obstruction, airway eosinophilic inflammation and bronchial hyperresponsiveness (Djukanovic et al., 1990) and is a global health problem that results from a complex interplay between genetic and environmental factors (Phillip, 2003), and excess production of Th2 cytokines (IL-4, IL-5, IL-13) relative to Th1 cytokine (IFN- γ).

Several medicinal herbs have shown to promote immunity in different ways. They have shown to augment specific cellular and humoral immune response. *Liriope platyphylla* is a medicinal herb that has been used for treating cough and

sputum in Korea. Jinyoung et al. (2004) reported that butanol fraction of *Liriope platyphylla* induced nerve growth factor in C6 and primary astrocytes cells. Moreover, Tai et al. (2002) reported that it improves airway mucociliary clearance and that the improvement may, at least in part, be ascribed to the amelioration of airway mucus secretion.

However, no study on the anti-asthmatic and anti-inflammatory activity of *Liriope platyphylla* has been reported. In order to verify the anecdotal claims that *Liriope platyphylla* has numerous phytochemical and herbological benefits, we have investigated the immunomodulatory and anti-inflammatory activities of the plant. The aim of this study was to evaluate the control activity of *Liriope platyphylla* extract on Th1- and Th2-type cytokines and other factors. Therefore, we decided to investigate anti-inflammatory and anti-asthmatic effects of *Liriope platyphylla* extract in murine model of asthma. The effects of *Liriope platyphylla* extract

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on OVA-induced BALF cells proliferation, cytokine genes expression, flow cytometric analyses and cytokines production were determined.

2. Materials and methods

2.1. Plant material and preparation of extracts

The sample of the fruits of *Liriope platyphylla* were purchased from Oriental Medical Hospital (Daejeon, Korea) in August, 2003. The plant was identified by Professor Young-Cheol Lee, College of Oriental Medicine, Sangji University in Wonju, Korea and a voucher specimens (LRT) are deposited in our laboratory (Department of Herbol-ogy, College of Oriental Medicine, Sangji University Wonju 220–702, Republic of Korea). Plant material (200 g) was extracted three times with H₂O. Then, the extract was filtered and evaporated on a rotatory evaporator (Rotary evaporator, BUCHI B-480, Switzerland) and finally dried by a freeze drier (Freeze dryer, EYELA FDU-540, Japan) to yield the extract LRT (30 g). The yield (w/w) of the extract was about 15%.

2.2. Animals

Seven to 8-week-old male C57BL/6 mice were obtained at Daehan Biolink Co. LTD. (Eumsung, Republic of Korea). They were maintained in an air-conditioned room with light from 7 a.m. to 7 p.m. The room temperature (about $22 \pm 1^\circ\text{C}$) and humidity (about $55 \pm 10\%$) were controlled automatically. Laboratory pellet chow (Samyang Inc.) and water were given ad libitum.

2.3. Digestion of pulmonary tissue and cells preparations

Single cell suspensions from lung tissues and BALF were isolated by mechanical disruption in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 50 μM 2-mercaptoethanol, 20 mM HEPES, and 2% heat-inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY). Briefly, Lungs were subsequently removed from thoracic cavity. After mincing using sterile scalpels, tissue was incubated in PBS containing 1 mg/ml Collagenase IV and 2 mg/ml Dispase II for 40 min at 37°C in a sterile polypropylene tube. After incubation, lung tissue was vigorously pipetted up and down to further dissolve remaining tissue clumps and then filtered using 70 μm cell-strainer (Falcon, Le Pont de Claix, France). Total cells of each samples were counted.

2.4. Cell proliferation assay

Isolated eosinophils (2×10^5 cells/well) were plated on 96-well flat-bottom microtiter plates (Costar, Cambridge,

MA) in 100 μl of RPMI 1640 supplemented with 20% FBS, and 100 μl of LRT, rIL-3 (30 U/ml) or rIL-5 (10 $\mu\text{g/ml}$) anti-bodies were added. For the [^3H] thymidine uptake assay, cells were incubated for 72 h in a humidified 5% CO₂ incubator at 37°C . The cells were pulsed with 0.5 μCi of [^3H] thymi-dine (specific activity, 84.8 Ci/mmol; New England Nuclear, Boston, MA) for the last 6–16 h of incubation, and were then harvested onto a glass-fiber filter using an automated cell harvester (Inotech, Zurich, Switzerland). The amount of radioactivity incorporated into the DNA was determined using a liquid scintillation counter (LS 6000A; Beckman, Palo Alto, CA).

2.5. OVA sensitization and inhalation

OVA (500 $\mu\text{g/ml}$) in PBS was mixed with equal volumes of 10% (w/v) aluminum potassium sulfate (alum; Sigma) in distilled water. Then incubated for 60 min at RT after adjust-ment to pH 6.5 using 10N NaOH, and centrifuged at $750 \times g$ for 5 min. OVA/alum pellet was resuspended to the origi-nal volume in distilled water. All mice were immunized on two different days (21 days and 7 days before inhalational exposure) by i.p. injections of 0.2 ml alum-precipitated Ag containing 100 μg of OVA (Sigma–Aldrich Korea, Korea) bound to 4 mg of aluminum hydroxide (Sigma–Aldrich Korea, Korea) in PBS. Seven days after the second sensi-tization, mice were exposed to aerosolized ovalbumin for 30 min/day on 3 days a week for 12 weeks (at a flow rate of 250 l/min, 2.5°C ovalbumin in normal saline) and intra-tracheally injected 250 μg of OVA (on day 8) on the back of the tongue. LRT (150 mg/kg) were orally administered three times a week for last 8 weeks. One day after the last of the OVA exposures, samples (bronchoalveolar lavage (BAL) fluid, lung cells, and blood) were collected.

2.6. BALF

To evaluate airway inflammation, we experimented the accumulation of eosinophils in BALF. Mice were sacrificed with an intraperitoneal injection of sodium pentobarbitone (100 $\mu\text{g/kg}$). The trachea was cannulated and the left bronchi were tied for histological experiment.

Immediately after sacrifice, cells in the lungs were recovered by flushing 1 ml of BAL fluid (1 mM EDTA, 10% FBS, PBS) into the lungs via the trachea. Total cell counts were determined and 100 μl of fluid were cytospun onto glass slides using a Cytospin centrifuge (Cellspin, Hanil, Korea) (400 g for 4 min). Differential cell counts were performed after staining with Diff-Quik Stain Set (Baxter Healthcare Corp., Miami, Florida, USA). The supernatant of BALF was stored at -25°C for determination of cytokines.

2.7. Antibodies and flow cytometric analysis

All antibodies (CD3, CD4, CD8, CD69, CCR3, CD11b, Gr-1) for flow cytometric analysis were purchased from

Becton Dickinson (BD) PharMingen (San Diego, CA). Cells from lung tissues and BALF were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01% NaN₃) for 10 min on ice, and analyzed by two color flow cytometry on a FACScan using CellQuest software (BD Biosciences, Mountain View, CA).

2.8. RT-PCR

To study anti-asthmatic effects of *Liriopsis tuber* extract on cytokine gene expression from lung cells, RT-PCR was performed after quantitative normalization for each gene by a densitometry using β -actin gene expression. Briefly, total cellular RNA was extracted by using RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer's instruction. Aliquots (3 μ g) of total RNA were transcribed into cDNA at 37 °C for 1 h in a total volume of 20 μ l with 2.5 U of moloney murine leukemia virus reverse transcriptase (Roche, Mannheim, Germany). Reverse transcribed cDNA samples were added to a PCR mixture consisting of 10 $\times$ PCR buffer, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Takara, Tokyo, Japan), and 10 pmol of primers for each gene. The primer sequences are as follows: mouse IL-4, 5'-CCGTCGATAGTGGCATCCATGAAAC-3' and 5'-GGACCAATACCTGCTATAGGG-3' IL-5, 5'-AACCCCTTACTGAAGTCAGATTGTTAG-3 and 5'-TAAGTCAGTTTAATGCTTAGGG-3' IL-13, 5'-TTCAAATGAGATTGTGGGAAAT-3' and 5'-ACCGATACAGTACAGTACAGTA-3' IFN- γ , 5'-CCGATATTAGATACGTTAAAC-3' and 5'-ATGGCCTAGTCAGTCTCTAAAT-3' β -actin, 5'-TGGAATCTGTGGTCCATGAAAC-3' and 5'-GTCACAGTCAGCTGTATAGGG-3'. Amplifications were performed with 25 cycles for β -actin and 30 cycles for the others. The amplification profile included denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by an additional extension step at 72 °C for 10 min. PCR products were electrophoresed and visualized by ethidium bromide staining.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Interleukin (IFN- γ , IL-4, IL-5, IL-13, etc.) production from BALF and serum of the indicated mice ($n = 5$) was measured by ELISA according to the manufacturer's instruction on a monoclonal antibody-based mouse interleukin ELISA kit (R&D system). All data represent the standard deviation of at least three different determinants and were compared using Student's paired *t*-test.

2.10. H&E and Masson trichrome staining in murine OVA-induced asthma lung cells

C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of LRT extract for the later 8 weeks (three

times a week). At the end of the experiment, the mice lungs were removed and analyzed histology.

2.11. Statistical analysis

For statistical analysis of data, *p*-values were analyzed using a paired Student's *t*-test software program (Startview 5.1; Abacus Concepts, Berkeley, CA). Results were considered statistically significant when *p* values were $p < 0.05$, $p < 0.01$, $p < 0.001$ ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

3. Results

3.1. Inhibitory effect of LRT on BALF cells proliferation

To study the effects on BALF cells proliferation, IL-3 or IL-5 activated cells were treated with varying concentrations LRT respectively. As shown in Table 1, treatment with LRT for 3 days stimulated cell proliferation as reflected by the decrease in tritiated thymidine uptake.

The tritiated thymidine uptake in the LRT (100 μ g/ml; 8701 $\pm$ 582 cpm) or CsA stimulated states (4676 $\pm$ 972.7 cpm) were decreased significantly. The similar results were observed in other concentrations (10, 1 μ g/ml; 8655 $\pm$ 336 cpm, 9354 $\pm$ 811 cpm). Furthermore, the inhibitory effect of the LRT on activated BALF cells was concentration dependent in vitro experiment (Table 1).

3.2. Inhibitory effect of LRT on airway eosinophil accumulation

LRT also attenuated the decrease of eosinophils in BAL induced by repeated i.n. administration of allergen (OVA inhalation 500 μ g/ml; on two or three consecutive days). This effect was associated with some degree of inhibition of lung eosinophilia. The number of eosinophils was

Table 1
Effects of the LRT on BALF cells proliferation

Group	Drug (μ g/ml)	rIL-5 + rIL-3	Eosinophil proliferation (cpm)
Eosinophil cells	–	–	968 $\pm$ 91
Control	–	+	18847 $\pm$ 1527
Cyclosporin A (CsA)	10	+	4676 $\pm$ 972
	100	+	8701 $\pm$ 582
Liriopsis tuber (LRT)	10	+	8655 $\pm$ 336
	1	+	9354 $\pm$ 811

Cell proliferation was measured by [³H] thymidine uptake as described above in Section 2. Briefly, 2×10^5 BALF cells were cultured with or without rIL-3 (30 U/ml) or rIL-5 (10 μ g/ml) antibodies in the presence or absence indicated concentration of the LRT for 3 days. Then tritiated thymidine was pulse-labeled for 16 h. before harvest. Radioactivity was determined by a scintillation counter. Each point represents the mean of three independent experiments. Results are representative of three independent experiments and are expressed as the mean $\pm$ S.D. of triplicate cultures ($***p < 0.001$ vs. cpm of control group).

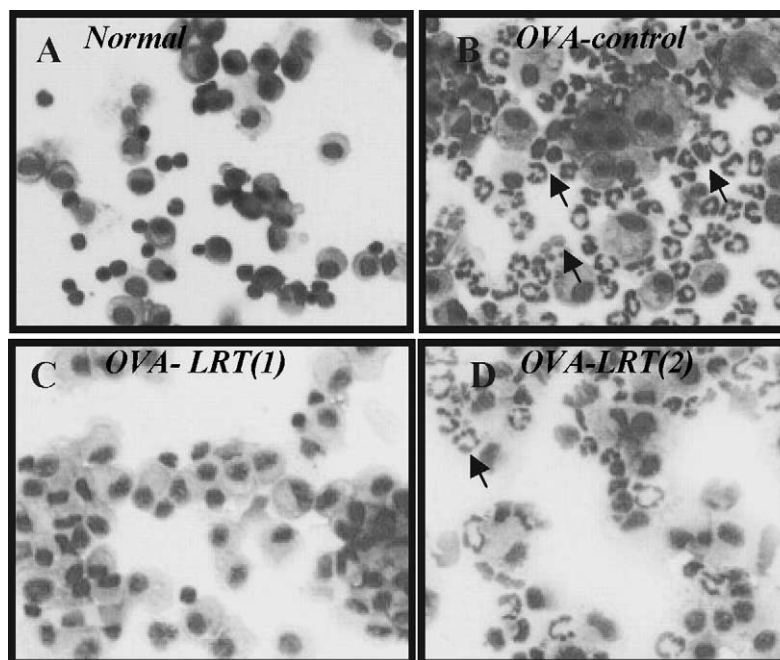


Fig. 1. Photomicrograph (original magnification, 400 $\times$) of BALF cytopins from a sensitized mouse exposed repeatedly to allergen (OVA; 500 μ g/ml, i.n. for 30 min/day on 3 days a week for 12 weeks at a flow rate of 250 l/min, 2.5% ovalbumin in normal saline). Normal: normal C57BL/6 mice OVA-control: ovalbumin inhalation (control) OVA + LRT (150 mg/kg); liriopis tuber (LRT); arrow: eosinophils.

significantly different among these three groups. In OVA-exposed group, the number of eosinophils was significantly increased, but in LRT treated group, the number of it was significantly decreased in comparison with the OVA-exposed group (Fig. 1).

3.3. Influx of inflammatory cells into airways after repeated allergen exposure

Intranasal administration of allergen (OVA inhalation 500 μ g/ml) to sensitized mice on two, or three consecutive days induced increases in mainly eosinophils in BAL, as compared with sensitized mice exposed to PBS (Fig. 2). The number of BAL eosinophils was sequentially increased after two or three consecutive days of OVA exposure. To evaluate the effect of LRT on the recruitment of cells to the airway, we investigated the lung weight, total lung cells, total leukocytes and eosinophils in BALF. Fig. 2 shows that total lung weight of LRT treated group was significantly reduced compared with OVA exposed control group, and the numbers of total leukocytes and eosinophils were also significantly reduced compared with that. Otherwise, the decrease number was not significant in total lung cells. This means that the relative number of eosinophils in relation to the total number of lung cells, especially granulocytes was a dominant portion.

3.3.1. Inhibition of cytokines production in BAL fluid and serum

To study whether LRT was related to inflammatory cytokines production, BALF supernatants were collected,

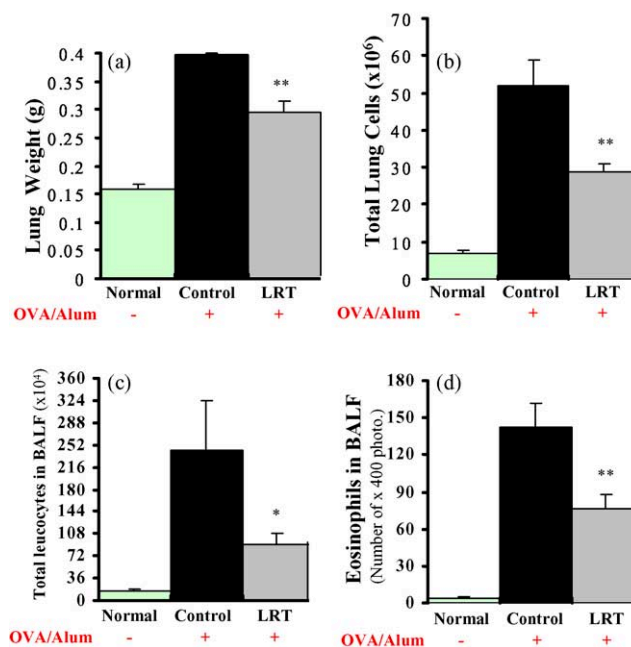


Fig. 2. Effect of LRT extract on total leukocytes number in BALF and lung cells in OVA-induced murine model of asthma. C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with LRT extract for the later 8 weeks. At the end of the experiment, the mice lungs were taken out and weighted. Then, the number of total leukocytes, eosinophils in BALF and lung were counted. Normal: normal C57BL/6 mice; OVA-control: ovalbumin inhalation (control); OVA + LRT (150 mg/kg); the results are expressed the mean $\pm$ S.E. (N = 5). Statistically significant value compared with control group data by *t*-test (* p < 0.05).

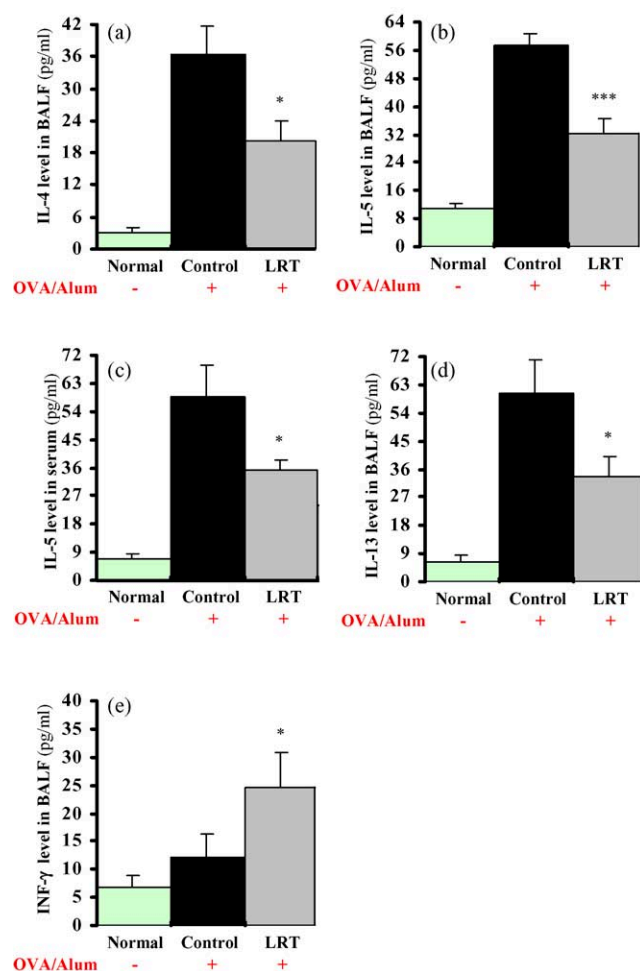


Fig. 3. Effect of LRT extract on cytokine level in BALF and serum in OVA-induced murine model of asthma. C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of LRT extract for the later 8 weeks. At the end of the experiment, the BALF and serum were collected and cytokine production were analyzed by ELISA. Normal: normal C57BL/6 mice; OVA-control: ovalbumin inhalation (control); OVA + LRT (150 mg/kg); the results are expressed the mean $\pm$ S.E. ($N=5$). Statistically significant value compared with control group data by t -test (* $p < 0.05$, ** $p < 0.01$).

and the productions of IL-4, IL-5, IL-13 and IFN- γ were analyzed by ELISA, respectively.

As shown in Fig. 3, IL-5 productions in activated BALF and serum were significantly suppressed by LRT. LRT enhanced IFN- γ productions in BALF cells (not significant), but in serum the IFN- γ productions was not detected.

Both IL-13 and IL-4 levels were similarly reduced in LRT treated groups of mice (not significant).

3.3.2. Measurement of IgE and histamine levels in BAL fluid and serum

To investigate the effect of LRT on IgE synthesis and histamine release, LRT (150 mg/kg) were orally administered three times a week for 8 weeks. LRT prevents IgE production in both BALF and serum, but not histamine release.

Because IgE levels in serum are dependent upon IL-4 and may be considered an additional index of Th2-cytokine secretion, we measured IgE in BALF and serum from mice in all groups. We found that IgE levels in serum from OVA-induced murine model of asthma were significantly increased compared with normal groups (PBS only). LRT treatment of these mice significantly inhibited the production of IgE. These results support the conclusion that LRT suppressed the generation of a Th2-type immune response in this animal model of asthma.

3.4. Determination of FACS analyses

3.4.1. Inhibitory effect of LRT on granulocytes population and CCR3 expression in murine OVA-induced asthma lung cells

To evaluate the efficacy of LRT treatment on CCR3 expression, we compared the effects of LRT on intracellular CCR3 expression in OVA-induced asthma lung cells by using flow cytometry. After administration with LRT and subsequent stimulation with OVA, 5–14% of the gated granulocytes identified as probable blasts on the basis of forward and side scatter plots (Fig. 5) were positive for CCR3 (Fig. 5). Only 10–23% of granulocytes expressed CCR3 (Fig. 5), representing a reduction of approximately 7% in the level of expression of CCR3 compared with that seen in control cells, which were

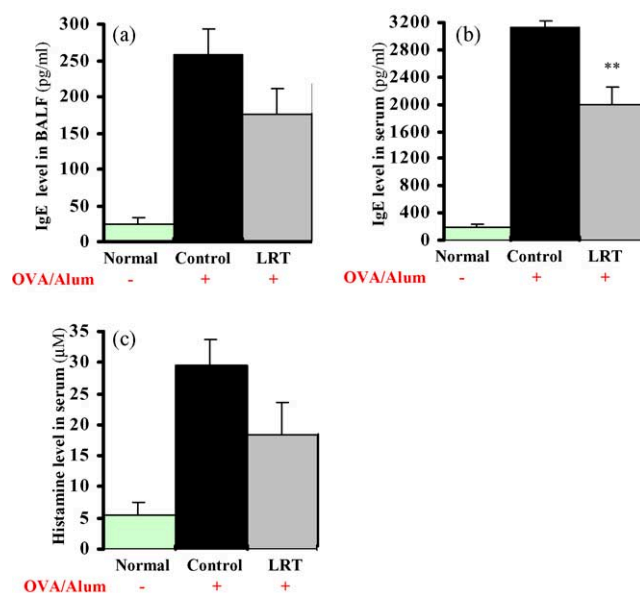


Fig. 4. Effect of LRT extract on immunoglobulin E level in BALF and serum in OVA-induced murine model of asthma. C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of LRT extract for the later 8 weeks. At the end of the experiment, BALF and serum were collected (Section 2) and analyzed by ELISA. Normal: normal C57BL/6 mice; OVA-control: ovalbumin inhalation (control); OVA + LRT (150 mg/kg); the results are expressed the mean $\pm$ S.E. ($N=5$). Statistically significant value compared with control group data by t -test (* $p < 0.05$, ** $p < 0.01$).

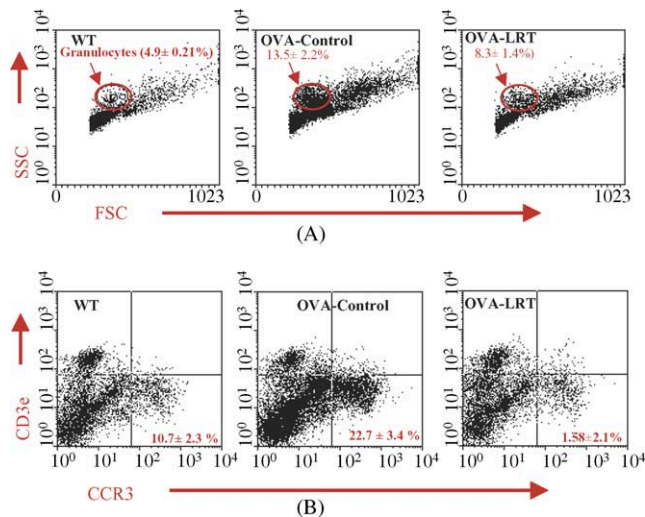


Fig. 5. Quantification by means of FACS analysis of granulocytes and eosinophils in lung cells: (a) shows the gated population of granulocytes determined on the basis of their side scatter (SSC) and forward scatter (FSC) and (b) shows the number of CCR3 expressing granulocytes (especially eosinophils) after sensitization and inhalation with OVA (shown in Section 2).

immunized with OVA (Fig. 5). Pretreatment with LRT had a small effect, decreasing by 7% the number of CCR3 expressing granulocytes compared with control cells (Fig. 5). The difference between control and LRT treatments was significant ($n = 5$, $p < 0.1$).

3.4.2. Inhibitory effect of LRT on lung cells (especially T cells) surface marker

Effects of LRT on leukocyte subsets in lungs, there were marked change in numbers of CD4⁺ and CD8⁺ T cells in lung of murine model of asthma compared to control cells, and the deficits in CD4⁺ and CD8⁺ T cells were accompanied by compensatory increases in proportions of B cells, regulatory T cells (Fig. 6). LRT treated group with OVA resulted in further significant reductions in CD4⁺CD8[−] T cells (by 11.4%), CD3⁺CD69⁺ (by 4.5%), Gr-1 (by 5.4%), CD11b (by 16.2%) and CD3⁺CD19[−] (by 29.4%) in lungs, whereas CD4⁺CD8⁺T cells, CD3⁺CD19⁺B cells and CD4⁺CD25⁺ regulatory T cells were reciprocally increased in these tissue (Fig. 6).

3.5. RT-PCR

To investigate whether the effects of LRT on cytokines production in BALF were related to cytokines mRNA expression in lung cells, total cellular RNA was extracted from lung cells treated with or without LRT in the presence or absence of OVA sensitization and inhalation and available for RT-PCR for cytokines. The amplification was determined by performing PCR for 30 cycles. As shown in Fig. 7, the mRNA for β -actin was detectable in the lung cells treated with PBS (Lane 1), OVA (Lane 2), OVA and LRT (Lane 3), respectively.

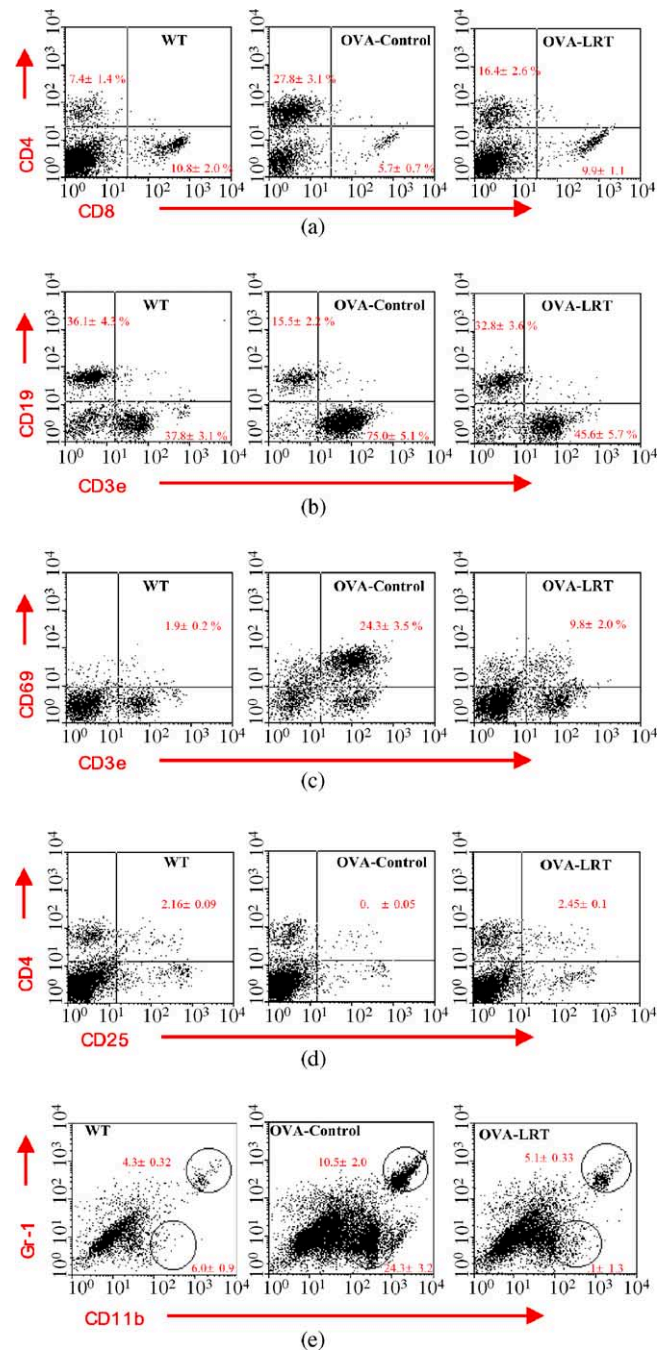


Fig. 6. Effect of LRT extract drug on lung cells surface markers in OVA-induced murine model of asthma C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of LRT extract for the later 8 weeks (three times a week). At the end of the experiment, the mice lungs were removed and analyzed by flow cytometer; WT: normal C57BL/6 mice, OVA-C: ovalbumin inhalation (control); OVA-LRT: OVA + LRT (150 mg/kg).

PCR products for IL-1 β , IL-4 and IL-5, amplified from lung cell RNA preparations, were decreased in the LRT treated groups compared with control groups (ova-induced asthma model mice group). Furthermore the LRT had little effects on IL-13, IFN- γ mRNA expression in lung cells. The

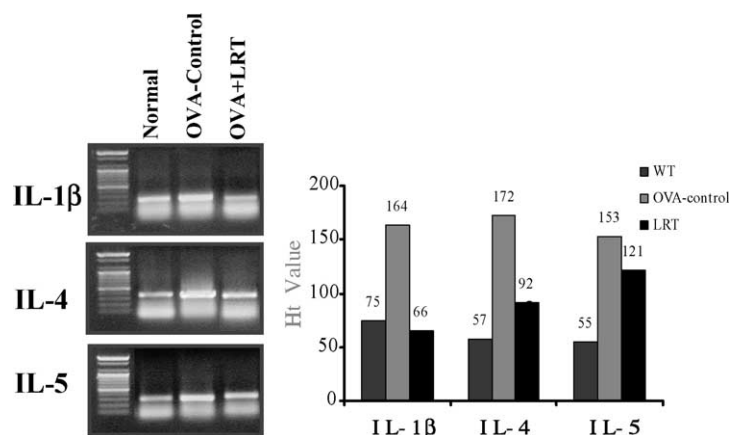


Fig. 7. Effect of LRT extract on IL-1 β , IL-4, and IL-5 mRNA gene expression in lung tissue of OVA-induced murine model of asthma C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of LRT extract for the later 8 weeks (three times a week). At the end of the experiment, the mice lungs were removed and analyzed RT-PCR. Normal: normal C57BL/6 mice; OVA-control: ovalbumin inhalation (control); OVA-LRT: OVA + LRT (150 mg/kg).

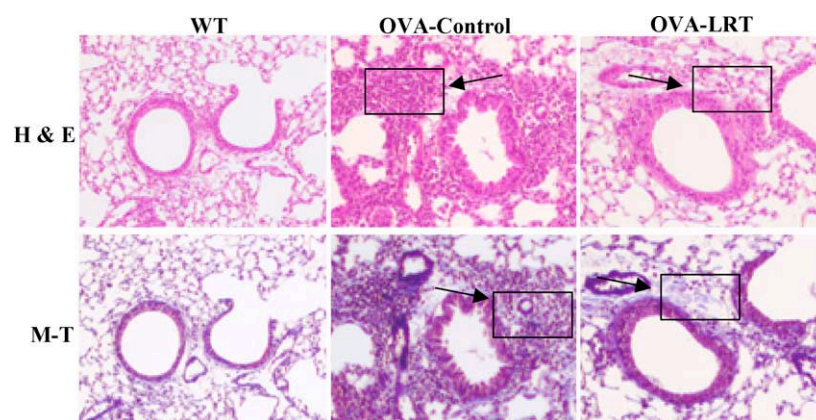


Fig. 8. Effect of LRT extract drug on histology of lung tissue (H&E and Masson trichrome staining) in lung cells of OVA-induced murine model of asthma C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks (three times a week) for asthma induction. Two experimental groups were treated with different concentrations of LRT extract for the later 8 weeks (three times a week). At the end of the experiment, the mice lungs were removed and analysed histology. H&E: hematoxyline–eosin stain; WT: normal C57BL/6 mice; OVA-control: ovalbumin inhalation; OVA-LRT: OVA + LRT (150 mg/kg).

results demonstrated that LRT significantly affected IL-1 β , IL-4 and IL-5 mRNAs expression but not other cytokines in lung cells.

3.6. Histological analyses of lung sections from OVA-induced asthma model mice after final antigen challenge

To clarify the efficacy of LRT on lung cells of murine asthma model, the left lungs were histologically examined 24 h after the final antigen challenge. Histological analyses of lungs from PBS-exposed sensitized mice showed normal lung histology (Fig. 8). In contrast, similar to the BALF study, histological sections of lung tissue from OVA-exposed mice exhibited airway inflammation. Infiltrating eosinophils were chiefly observed in the peribronchial regions of the lung. While on the other hand, exhibition of airway inflammation was decreased in histological sections of lung tissue from LRT treated mice (Fig. 8).

4. Discussion

One of the therapeutic objectives in asthma is to reduce the regional inflammatory response through the reduction of antigen-induced inflammatory cells and inflammatory cytokines production. The main question to be addressed is whether LRT are involved in the pathology of airway inflammation and asthma. In the present study, we examined lung cells inflammation and BALF analyses.

Bronchial asthma is a chronic inflammatory disorder of the airways characterized by various airway obstruction (mucus production, peribronchial thickening, fibrosis, etc.), airway eosinophilic inflammation and bronchial hyperresponsiveness. It is a global health problem that results from a complex interplay between genetic and environmental factors (Phillip, 2003).

Airway allergen challenge causes cellular inflammation in the airways, which is dominated by eosinophils in both humans (Kroegel et al., 1994) and mice (Ohkawara et al.,

1997). It has been suggested that eosinophils contribute to several of the clinical features of allergic asthma, including tissue damage and airway hyperresponsiveness (Lotvall et al., 1998). Furthermore, eosinophils also have the capacity to release substantial amounts of bronchoconstrictor mediators, such as leukotrienes (Owen et al., 1987). The use of mice BALF cells offers a popular system for the study of asthma pathogenic mechanisms. Although the involvement of other inflammatory mediators cannot be excluded, OVA-induced elicitation of these inflammatory factors, which are released by BALF cells located in the airway, probably play a crucial role in the pathogenesis of asthma (Schwartz et al., 1994).

In our study, the proliferation and various cytokines production of BALF cells from OVA-induced control subjects could be enhanced, but LRT-treated subjects significantly reduced. In many cases, IFN- γ enhances IgG2a production and inhibits IgG1 and IgE production in B lymphocytes. IFN- γ may induce terminal differentiation of macrophages and monocytic cell lines and therefore curtail cells proliferation. IL-10 actively suppresses the development of the TH1 lymphocytes and enhances B cells proliferation and IgE synthesis (Kim et al., 1992). IL-1 β , a pro-inflammatory cytokine involved in a variety of immune responses, can promote mast cell activation and production of Th2 cytokines (Hultner et al., 2000; Stassen et al., 2000), suggesting that IL-1 functions in the development of allergic diseases. IL-4 is indeed able to induce the rolling on and adhesion of circulating eosinophils to endothelial cells, which can then be attracted into target tissues by both IL-5 and chemokines (Sergio, 2001). Also, IL-4 and IL-13 are required for AHR induction through inducing allergen-specific IgG1 and IgE production (Bochner and Schleimer, 1994). IL-5 has been shown to be crucial for the induction of airway eosinophilia, because mice lacking IL-5 (IL-5 knockout mice) fail to develop airway eosinophilia in response to allergen exposure unless the animals are reconstituted with IL-5 (Wang et al., 1998).

Therefore, we suggest that the inhibitory mechanisms of LRT on BALF cells proliferation and IL-1 β , IL-4, IL-5, IL-13, and IFN- γ production may have involved: (a) the LRT stimulated IFN- γ mRNAs expression in OVA-induced BALF cells; (b) enhanced secretion of IFN- γ may inhibit the IL-10 mRNA expression and IL-10 production in the cells; (c) reduced production of IL-5, IL-4 and IL-13 will suppress the BALF cells proliferation (Table 1); (d) then more factors synthesized by LRT treated BALF cells and serum such as IgE, histamine were decreased (Fig. 4). However, LRT how to regulate IL-5 and IFN- γ genes expression in OVA-induced and LRT treated BALF cells and serum is still unknown. The mechanisms of action are subjects for further study.

We predict that impairments of IL-1 β , IL-4, IL-5 and IFN- γ production in lung cells treated with LRT may be related to decreasing in BALF cell, total lung cells, and granulocytes numbers in lung tissue. We found that LRT affected IL-1 β , IL-4, IL-5 and IFN- γ production in BALF cells and lung cells through modulation mRNA expression. Although the

RT-PCR is not so strong in quantitative determination, and it is a quick method to detect mRNA. In this experiment, the β -actin mRNA was used as an internal control then the ratio of each cytokine to β -actin mRNA was statistically analyzed.

CCR3 receptors are expressed on basophils, eosinophils, mast cells, and Th2 cells; CCR2, CCR4, and CCR8 are expressed on T-helper lymphocytes (Homer and Boushey, 2003). It is of note that the eotaxin receptor [CC chemokine receptor (CCR)3] has been described not only on eosinophils (Heath et al., 1997) and basophils, but also on Th2 cells themselves. Our results indicate that LRT could decrease CCR3 expression of BALF cells (especially eosinophils) which data are compatible with cell proliferation and airway eosinophil accumulation results.

The CD11b expression on neutrophils and eosinophils was significantly higher in asthmatic children than in the controls. It is investigated that the inducible expression of CD11b on neutrophils and eosinophils from allergic asthmatic children is primed in vivo (Berends et al., 1993). CD11b expression on the surface of circulating eosinophils is significantly elevated in various allergic disorders, including atopic dermatitis and bronchial asthma (Yachie et al., 1993).

We observed that inhalation challenge with LRT administration resulted in a decrease in airway CD11b+ macrophage, Gr-1 granulocytes when compared with that observed after only OVA challenge. We also observed that the change in the number of eosinophils present compared with only OVA challenge correlated with the change in BALF cell proliferations.

It has recently been shown that CD25 is expressed more highly on peripheral blood than on BAL lymphocytes, whereas the opposite was found for CD69 (Ekberg-Jansson et al., 1999).

CD4+CD25+ regulatory T cells express the IL-2 receptor surface marker CD25 and constitute about 10% of the CD4+ T cells. Their role in preventing autoimmune conditions, transplant rejection, and inflammatory bowel disease has been established (Javier Chinen et al., 2004). Umetsu et al. (2003) reviewed the role of different immune regulatory cells in allergic disease and asthma, including CD25+CD4+ T cells. Mouse models and data from human beings suggest that these regulatory cells and TH3 cells (CD4+ T cells that produce TGF- β) are important in the control of asthma, reducing the inflammation induced by cytokines and immune cell activation. Bellinghausen et al. (2003) showed that CD4+CD25+ cells from seven atopic patients and six nonatopic patients equally inhibited TH and TH2 responses, and although these cells were equally present in atopic and nonatopic patients, a subset of the atopic patients had regulatory cells that predominantly suppressed only TH1 responses.

In our study, CCR3 expression on granulocytes is down-regulated by LRT. Because the expression of CCR3 could be affected by cytokines, we further examined by immunostaining the expression of different cell surface markers. As shown in Figs. 4 and 5, LRT enhanced CD8+, CD19+, CD25+ expression on lung cells, while activation with Th1 cytokines

(IFN- γ) resulted in a significant decrease in the expression of CCR3, CD4, CD69 in lung cells.

If IL-4, IL-5, IL-13 predominates, CD4 precursor cells become type 2, T-helper cells (Th2) that secrete IL-4, IL-5, and IL-13. These Th2 cytokines cause recruitment of eosinophils to the airway. Binding and subsequent antigen-dependent cross-linking of IgE bound to Fc RI receptors on eosinophils results in eosinophil activation and degranulation. Local release of mediators from eosinophils such as histamine cause injury to the airway in the form of epithelial cell sloughing, increased mucous production, and airway hyperreactivity (AHR).

The therapeutic activity of LRT on asthma in oriental medicine may be partly related to: (a) immunomodulatory agents contained in LRT enhance the TH1 immune responses such as IFN- γ productions then TH2 cells immune responses will be decreased; (b) the TH1 immune responses will inhibit IgE production in B lymphocytes; (c) reduced production of IgE will attenuate asthma attack occurring in individuals.

In summary, LRT has a deep inhibitory effect on airway inflammation and hyperresponsiveness in murine model of asthma and that suppression of IL-5, IgE, eosinophil CCR3 expression and CD11b expression, increasing of IFN- γ production in BALF contributes this effect. Hence, the results indicated that LRT could act as a immunomodulator which possess anti-inflammatory and anti-asthmatic property also by modulating the relationship between Th1/Th2 cytokine imbalance. LRT appear to be a potential therapeutic herb medicine for treating airway inflammation and hyperresponsiveness in murine model of asthma.

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