

Effects of lactose- β -sitosterol and β -sitosterol on ovalbumin-induced lung inflammation in actively sensitized mice

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Abstract

Asthma is a disease marked by chronic lung inflammation and the number of patients suffering from asthma increases annually. Both β -sitosterol (BS) and β -sitosterol glucoside exist in a variety of plants and have anti-tumor, anti-microbial, and immunomodulatory activities. However, the precise role of BS and β -sitosterol glucoside in asthma has not been well understood. The aim of this study was to investigate the inhibitory effects of BS and lactose-BS (L-BS) on the pathophysiological process in ovalbumin-induced asthmatic mice. The total cells and eosinophils in the bronchoalveolar lavage (BAL) fluid markedly decreased ($p < 0.05$) after L-BS or BS administration (1 mg/kg; i.p.), and the ROS production also decreased in comparison to the asthma control. Histopathological features were detected by performing histochemistry, including H&E and alcian blue & P.A.S staining. Both L-BS and BS mitigated the inflammation by eosinophil infiltration and mucus hypersecretion by goblet hyperplasia. These effects of L-BS were superior to those of BS. L-BS and BS inhibited the increased mRNA and protein expression of IL-4 and IL-5 in the lung tissue and BAL fluid, respectively. The IgE concentration in the BAL fluid and serum was measured by performing ELISA and the ovalbumin-specific IgE in the BAL fluid was uniquely inhibited by L-BS ($p < 0.05$). The splenocytes were isolated from the normal and asthmatic mice and incubated in the absence and presence of 100 μ g/ml ovalbumin, respectively. L-BS blocked the survival rate of the splenocytes of the mice ($p < 0.01$). This finding indicates the possibility of L-BS and BS as potential therapeutic molecules in asthma and may contribute to the need to improve current therapeutic drugs.

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1. Introduction

Asthma is an allergic disorder of the respiratory system characterized by the excessive infiltration of leukocytes

into the lung, particularly into eosinophils, bronchoconstriction, increased airway hyperresponsiveness, and mucus production [1]. This complex disease is involved in a variety of cell types including mast cells, eosinophils, and the type 2 T-helper (Th2) lymphocytes. An imbalance between Th1 and Th2 lymphocytes contributes to the onset of the disease, the progression, and continuance of the disease. The elevated release of Th2 cytokines, IL-4, IL-5, and IL-13 has been known to induce IgE switching in B cells, the recruitment of mast cells and eosinophils,

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and the production of various inflammatory mediators [2]. Eosinophils are known as the primary effector cells in the pathogenesis of asthma by the release of specific granule proteins and reactive oxygen species [3]. The severity and mortality of asthma are rapidly elevating around the world, specifically in developed countries, and 3–10% of the world's population suffers from asthma [4]. Several drugs for asthma therapy are currently available; however, most of them are inadequate and have adverse side effects.

β -sitosterol (BS) is one of the phytosterols present in a large number of plants and its concentration is detected at low levels in the serum and tissues of healthy people eating fruits and vegetables [5]. BS glucoside is a glucosylated form of BS and is found in numerous plants. It has been reported that BS and BS glucoside regulate the

proliferation and activities of peripheral blood lymphocytes and NK cells, blood cholesterol level, anti-pyretic activity, and insulin release [6–9]. These reports indicate that BS and BS glucoside have possible therapeutic effects on carcinogenesis, cardiovascular disease, pulmonary tuberculosis and immune-mediated disorders [10]. BS and BS glucoside are believed to be important molecules in immune-associated abnormalities such as allergic inflammation. However, the effect of BS or BS glucoside on asthma remains to be determined. In this respect, we hypothesized that BS or lactose-BS (L-BS), a BS glucoside, has a therapeutic effect on asthma. Since L-BS has a higher solubility than BS, we thought that L-BS would have a better effect on the inhibition of asthma. We investigated whether BS or L-BS mitigates bronchus

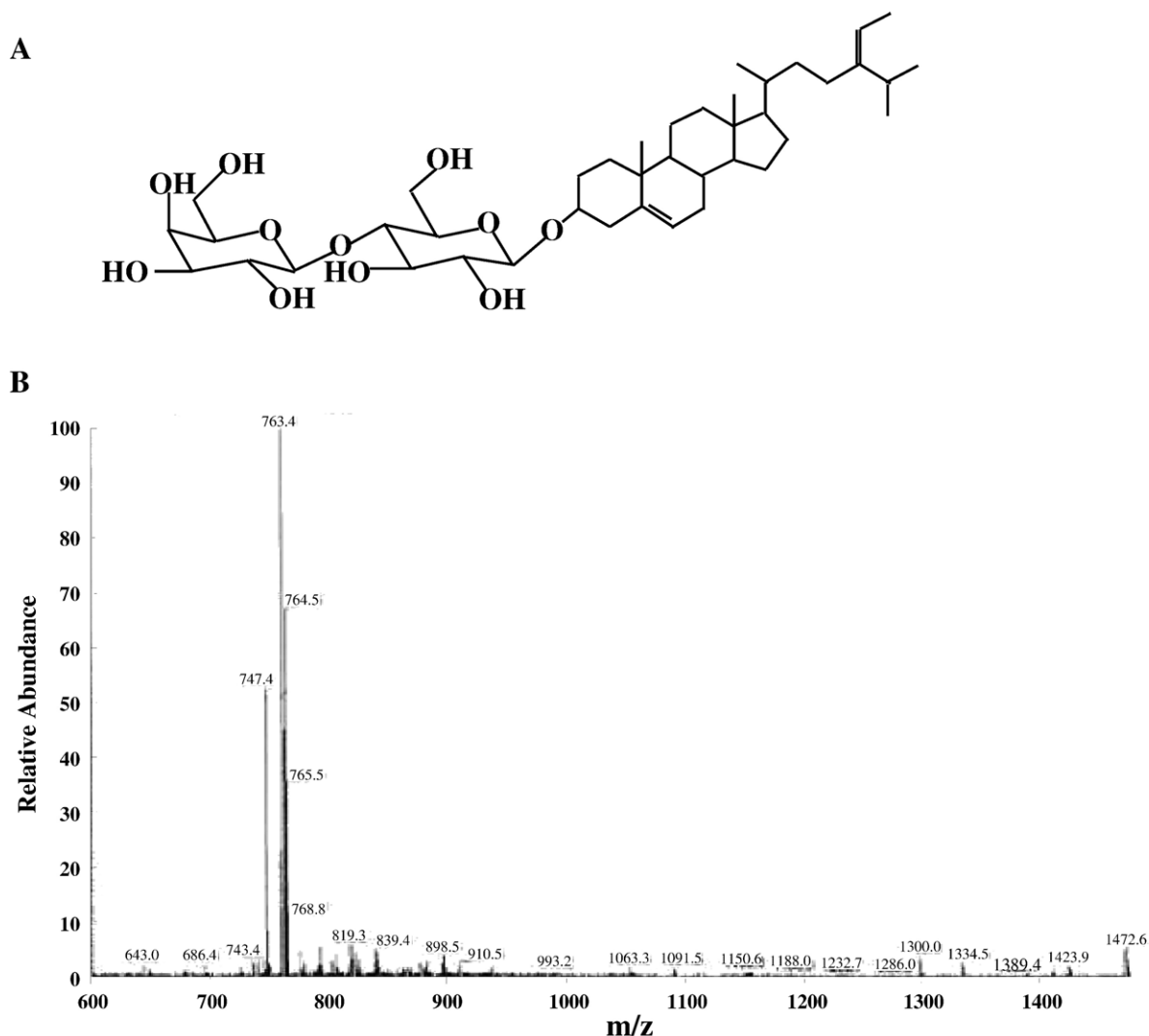


Fig. 1. Chemical structure (A) and LC-MS chromatogram (B) of L-BS. L-BS was synthesized from BS as thoroughly described in the Materials and methods section.

constriction, eosinophil infiltration, mucus production and protein level, including Th2 cytokines and IgE in an asthmatic animal model induced by ovalbumin and we examined the comparative effects between BS and L-BS.

2. Materials and methods

2.1. Synthesis of L-BS

L-BS was prepared using several chemical reactions. The synthesis of *o*-peracetyl lactose was obtained by an ethyl acetate (J. T. Baker, Phillipsburg NJ) extraction after reacting distilled lactose (Sigma-Aldrich Korea, Yongjin, Korea) with acetic anhydride (Sigma-Aldrich Korea). 1-bromo-*o*-peracetyl saccharide prepared from *o*-peracetyl lactose reacted with BS (Sigma-Aldrich Korea) in the presence of boron trifluoride etherate (Sigma-Aldrich Korea), resulting in the production of *o*-peracetylsaccharide- β -sitosterol. Finally, a reaction of the chemical dissolved in methylene chloride (J.T.Baker) with KOH/MeOH (Sigma-Aldrich Korea) yielded the target compound L-BS. The structure was confirmed by ¹H NMR (Varian, Palo Alto, CA) and LC-MS (LC-MS buffer, MeOH 100% (direct injection); Thermo Finnigan AQA) (Fig. 1).

2.2. Induction of asthma in mice

Six to eight-week-old female BALB/c mice were obtained from Daehan Biolink Co. LTD (Seoul, Korea). They were maintained in an air-conditioned room. The room temperature (about 22±1 °C) and humidity (about 55±10%) were automatically controlled. The mice were divided into five groups (*n*=5), and airway inflammation was induced in four groups. Each mouse was immunized through intraperitoneal (i.p.) injection with 20 µg of chicken OVA (Grade III; Sigma-Aldrich Korea) and 1 mg of aluminum hydroxide (Sigma-Aldrich Korea) on days 1 and 14, as shown in Fig. 2. The mice were exposed to a 5% ovalbumin solution aerosolized using an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan) for 1 h per day from days 21 to 27 after the second sensitization. The mice were placed in a Plexiglass chamber (30×30×15 cm³) that contained small ventilation holes on one side during the inhalation challenge. The aerosol was generated with a nebulization rate of 1 ml/min. Three groups of asthma-induced mice were treated through i.p. injection with 1 mg/kg of L-BS, BS (Sigma-Korea) or dexamethasone (Sigma-Aldrich Korea) between days 14 and 27. Both L-BS and BS dissolved in DMSO diluted less than 1/100 by phosphate-buffered saline (PBS). The negative control group was sensitized and challenged with PBS without drug administration.

2.3. Collection of bronchoalveolar lavage (BAL) fluid

Collection of BAL fluid was performed by flushing 1 ml of PBS into the lungs via the trachea immediately after the sacrifice of the mice and approximately 0.7 ml of BAL fluid was recovered after 5 lavages. The BAL fluid was centrifuged

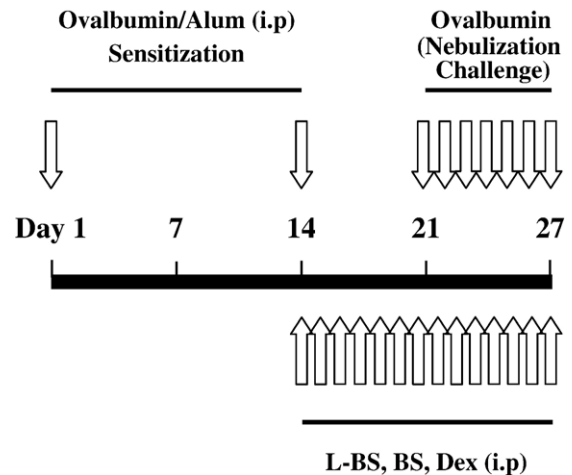


Fig. 2. Schematic diagram of an experimental protocol in mice. The mice were divided into five groups, and airway inflammation was induced in four groups. They were immunized through intraperitoneal (i.p.) injection with 20 µg of chicken OVA and 1 mg of aluminum hydroxide on days 1 and 14. The mice were exposed to a 5% ovalbumin solution aerosolized using an ultrasonic nebulizer for 1 h per day from days 21 to 27 after the second sensitization. Three of the asthma control groups were treated through intraperitoneal (i.p.) injection with 20 µg of lactose- β -sitosterol (L-BS), β -sitosterol (BS) or dexamethasone (Dex) between days 14 and 27. The negative control group was sensitized and challenged with PBS without drug administration.

(400×g, 4 °C, 5 min) and the supernatant was stored at –70 °C for the measurement of cytokines. The cells in the BAL fluid were washed three times with PBS and the pellet was resuspended in 100 µl of PBS. Total cell number was counted by Neubauer hemocytometer and a differential cell count was performed after the staining of cytopspined cells with a Diff-Quik Stain Set (Baxter Healthcare Corp., Miami, FL). The cells were differentiated by general leucocyte morphology and 200 cells per cytopspin preparation were counted. The percentage of each leucocyte was determined and absolute leucocyte count was calculated by multiplying each leucocyte percentage by total cell count.

2.4. Measurement of intracellular reactive oxygen species (ROS)

The cells in the BAL fluid were washed with PBS. The cells were incubated for 10 min at room temperature with PBS containing 3.3 µM 2',7'-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR) to label the intracellular ROS and were then immediately observed using fluorescence-activated cell sorting (FACS) analysis (Beckton Dickinson, San Jose, CA).

2.5. Histological analysis

Lung tissues were separated from the mice and fixed overnight with Carnoy's solution. The tissues were dehydrated at different concentrations of ethanol, embedded in paraffin, and

cut into 4 μm sections with a microtome (Leica, Nussloch, Germany). The sections were placed on glass slides, deparaffinized and stained with hematoxylin and eosin (Sigma-Korea) in order to examine the infiltrated cells or with alcian blue and periodic acid-schiff (Sigma-Korea) to evaluate the mucus production. A peribronchial cell count based on a five point scoring system was performed as previously described in order to estimate the severity of leukocyte infiltration [11]. The scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells higher than 4 cells deep. Goblet cell hyperplasia in the airway epithelium was performed using a five point grading system as previously described to estimate the extent of mucus production [12]. The grading system was: 0, no goblet cells; 1, lower than 25%; 2, 25–50%; 3, 50–75%; 4, higher than 75%. The scoring of the leukocytes and goblet cells was examined in three independent fields of lung section from each mouse.

2.6. Semi-quantitative RT-PCR

RT-PCR was performed to measure the relative quantities of mRNA for IL-4, IL-5, IL-13, IFN- γ , and eotaxin in the lung. The total RNA was extracted from the cells using Trizol reagent as described in the manufacturer's instructions. The total RNA (2 μg) was incubated at 37 °C for 90 min using the first-strand cDNA synthesis kit (Promega, Madison, WI) in order to prepare the cDNA. The primer sequences were as follows: mouse IL-4, 5'-TCT CTA GAT CAT GGG CAT TTT GAA CGA GGT C-3' and 5'-TGC ATG ATG CTC TTT AGG CTT TCC-3'; mouse IL-5, 5'-ATG ACT GTG CCT CTG TGC CTG GAG C-3' and 5'-CTG TTT TTC CTG GAG TAA ACT GGG G-3'; mouse IL-13, 5'-CTC CCT CTG ACC CTT AAG GAG-3' and 5'-GAA GGG GCC GTG GCG AAA CAG-3'; mouse eotaxin, 5'-AGA GGC TGA GAT CCA AGC AG-3' and 5'-CAG ATC TCT TTG CCC AAC CT-3'; mouse IFN- γ , 5'-TGC ATC TTG GCT TTG CAG CTC TTC CTC ATG GC-3' and 5'-TGG ACC TGT GGG TTG TTG ACC TCA AAC TTG GC-3'; mouse β -actin, 5'-AGG CTG TGC TGT CCC TGT ATG C-3' and 5'-ACC CAA GAA GGA AGG CTG GAA A-3'. PCR was carried out with an initial denaturation at 94 °C for 5 min, then 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C (IL-4, IL-5, IL-13, eotaxin, and β -actin), or 70 °C (IFN- γ) for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. β -actin was used as an internal control for each PCR reaction. The final PCR products were separated on 1% agarose gels and then visualized by ethidium bromide staining. Densitometric analysis was performed using the Quantity One software (Bio-rad Laboratories, Hercules, CA).

2.7. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-4, IL-5, IL-13, IFN- γ , eotaxin and IgE in the BAL fluid or serum was measured by a sandwich ELISA using OptEIA Set mouse IL-4, IL-5, IFN- γ and IgE (BD Biosciences, San Diego, CA) and Quantikine kit IL-13 and eotaxin (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All assays were performed in trip-

licate. The concentration was calculated using a linear-regression equation obtained from the standard absorbance values.

2.8. Viability of mouse splenocytes

We performed MTT assay and apoptosis assay to determine the cell viability with using the MTT assay kit (Roche, Penzberg, Germany) and annexinV-fluorescein isothiocyanate (FITC) apoptosis detection kit (Beckton Dickinson, San Jose, CA). The splenocytes were isolated from the spleens of normal and asthmatic control mice by syringe pumping. After three washings with 10 ml of DMEM supplemented with antibiotics–antimycotics (Life Technologies, NY), the splenocytes were incubated with 3 ml of RBC lysis buffer (Sigma, MI) for 10 min at room temperature, and then this was washed twice with 10 ml of the wash medium. 2×10^5 splenocytes in 100 μl of DMEM medium containing 10% FBS were seeded onto a 96-well culture plate. L-BS, BS or dexamethasone (1 $\mu\text{g}/\text{ml}$) in the absence or presence of 100 $\mu\text{g}/\text{ml}$ ovalbumin was added to the individual well and then the plate was incubated for 48 h at 37 °C in a CO₂ incubator. After the addition of 10 μl of MTT solution in each well, the plate was incubated at 37 °C for 4 h in a CO₂ incubator and 100 μl of solubilization solution was added to each well for MTT assay. After 24 h incubation, the absorbance was measured by using an ELISA reader (Bio-Tek Instruments, VT) at 550 nm. For the apoptosis assay, the cells were harvested and resuspended in binding buffer. Annexin V-FITC and PI were added and incubated for 15 min at room temperature. The cells were analyzed by FACS sort cytofluorimeter using CellQuest software. Negative cells against annexin V and PI staining were considered as viable or non-apoptotic cells.

2.9. Statistical analysis

Data are expressed as the means $\pm$ S.E.M. The statistical differences were analyzed using the one-way ANOVA followed by Turkey test. The SPSS statistical software package (Version 10.0, Chicago, IL) was used for the statistical analysis. A significant value was defined as $p < 0.05$.

3. Results

3.1. L-BS and BS inhibit total leukocytes and eosinophilia in BAL fluid

We induced asthma in BALB/c using the protocol described in Fig. 2 and examined the alternation of total cells in the BAL fluid in order to estimate the effects of L-BS and BS on asthma. L-BS significantly blocked the leukocytosis, and absolute eosinophilia and lymphocytosis by asthma induction ($p < 0.01$) (Fig. 3A and B). The number of increased eosinophils decreased after L-BS treatment and the number of decreased macrophages increased after L-BS treatment as determined by performing % differential leukocyte counting (Fig. 3C). L-BS also decreased ROS production in the BAL fluid (Fig. 3D). The effect of BS was similar to that of L-BS in the results from the leukocyte counting, but the overall effects of L-BS were better

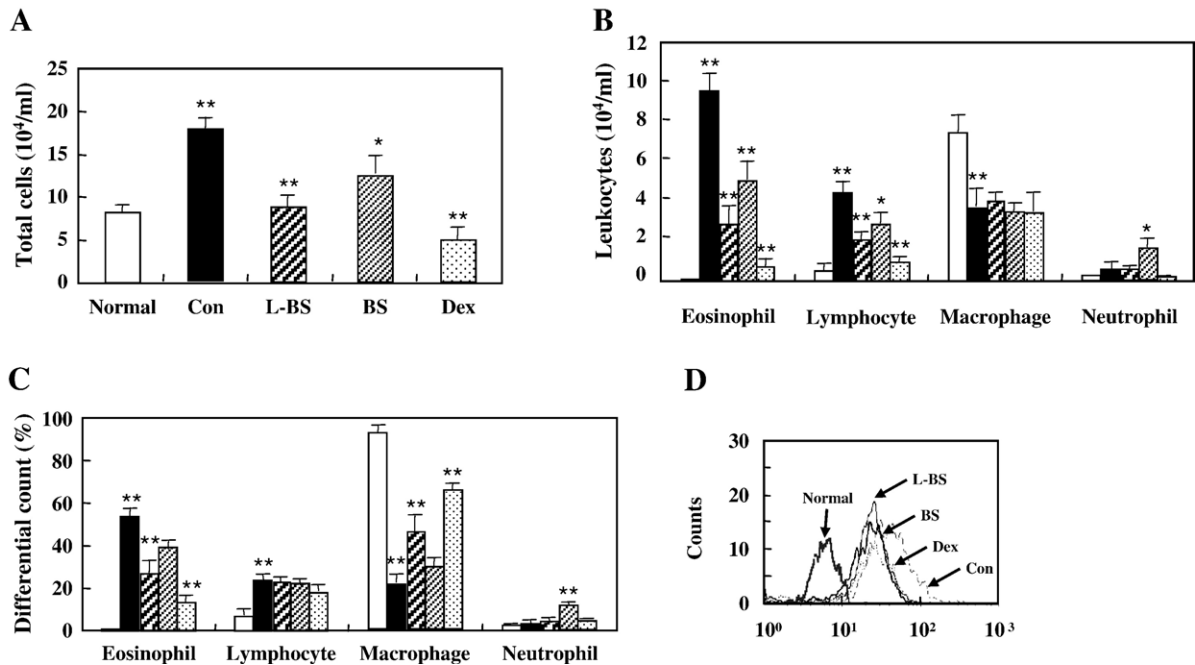


Fig. 3. Effect of L-BS and BS on the total and differential leukocytes in BAL fluid. BAL fluid was collected from ovalbumin-induced mice untreated (Con; closed bar) or treated with 20 μ g of lactose- β -sitosterol (L-BS; thick hatched bar), β -sitosterol (BS; thin hatched bar) or dexamethasone (Dex; dotted bar) between the sensitization and inhalation period. The negative control group (Normal; open bar) was sensitized and challenged with PBS without drug administration. The number of cells in the fluid was totally and differentially counted (A, B, C) following the centrifugation of the BAL fluid or the cells were incubated for 10 min at room temperature with PBS containing 3.3 μ M 2',7'-dichlorofluorescein diacetate (DCFDA) to measure the ROS level and were then immediately observed by fluorescence-activated cell sorting analysis (D). The results from the leukocyte counting in the cytopspined BAL fluid were expressed as the means $\pm$ S.E.M. of five independent experiments (A, B, C). * p < 0.05 and ** p < 0.01 are assessed as a significant difference between the normal group and asthma control group or between the asthma control group and drug-treated group.

than those of BS. These results indicate that L-BS and BS reduce lung inflammation in an asthma model.

3.2. L-BS and BS alter the histopathologic response of lung tissues

The asthmatic lung tissue induced by ovalbumin displayed a marked infiltration of inflammatory cells into the peribronchial connective tissues, hyperplasia and mucus generation of goblet cells within the bronchi, and disrupted the structure of the inter-alveolar septum as compared with the normal tissue (Fig. 4A and B). L-BS attenuated the increased inflammation and mucus score, representing eosinophil-rich leukocyte infiltration and mucus secretion (Fig. 4C). BS weakly mitigated the histopathologic expression. These results indicate that L-BS and BS reduce goblet cell hyperplasia and mucus production in the airway remodeling process.

3.3. L-BS and BS modulate cytokine expression in lung tissues

Since L-BS and BS inhibit the number of inflammatory cells in the BAL fluid, we next investigated both the mRNA and protein expression of the associated molecules. As shown

in Fig. 5A and B, L-BS and BS significantly inhibited the elevated mRNA and protein expression of IL-4 and IL-5 following asthma induction (p < 0.05) (Fig. 5). Despite the absence of significant value, BS weakly diminished the protein expression of IL-13 and eotaxin close to or lower than the normal condition and L-BS slightly increased the IFN- γ level as compared to the asthma control.

3.4. L-BS and BS decrease ovalbumin-specific IgE level in BAL fluid

We examined concentration of total IgE and ovalbumin-specific IgE in the serum and BAL fluid to further understand how L-BS and BS induce the inhibitory effects on asthma. Although L-BS has no effect on both levels in the serum, L-BS uniquely inhibited the level of ovalbumin-specific IgE in the BAL fluid (Fig. 6). BS weakly blocked ovalbumin-specific IgE in the BAL fluid without significant inhibition.

3.5. L-BS and BS inhibit the survival rate of mouse splenocytes

We next investigated the effects of L-BS and BS on the viability of mouse splenocytes by performing MTT assay,

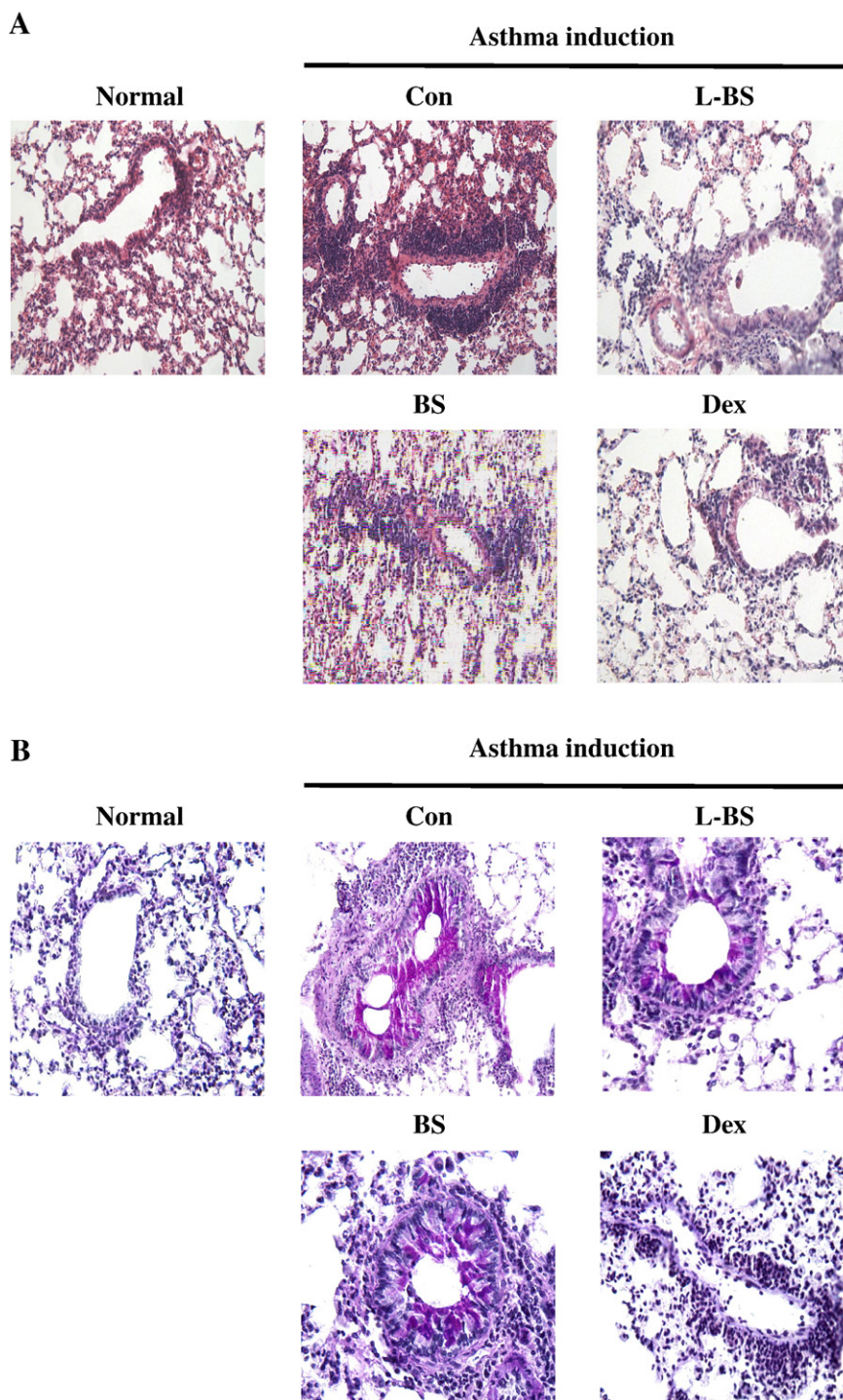


Fig. 4. Effect of L-BS and BS on airway inflammation and mucus hypersecretion in lung tissues. Lung tissues were excised from ovalbumin-induced mice untreated (Con) or treated with 20 μ g of lactose- β -sitosterol (L-BS), β -sitosterol (BS) or dexamethasone (Dex) between the sensitization and inhalation period. The negative control group (Normal) was sensitized and challenged with PBS without drug administration. The pathologic alternation of lung tissues was analyzed by histochemistry using H&E staining (A) or alcian blue & P.A.S staining (B) as described in the Methods section. Scoring of inflammatory cell infiltration (closed bar) and mucus production (open bar) in the lung tissues was performed as described in the Methods section (C). The scoring data are expressed as means $\pm$ S.E.M. of five independent experiments. * $p < 0.05$ and ** $p < 0.01$ are assessed as a significant difference between the normal group and asthma control group or between the asthma control group and drug-treated group. Magnification (A and B) $\times 200$.

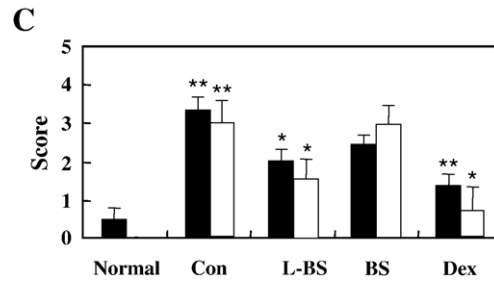


Fig. 4 (continued).

and annexin V-FITC and PI staining. The viability of the splenocytes increased after asthma induction, and that of the ovalbumin-treated cells elevated as compared with the untreated cells, despite the different effect between MTT assay

and annexin V-PI staining (Fig. 7). Both L-BS and BS blocked the survival rate of the splenocytes of the normal and asthmatic mice in the absence or presence of ovalbumin treatment. The effects of L-BS were better than those of BS.

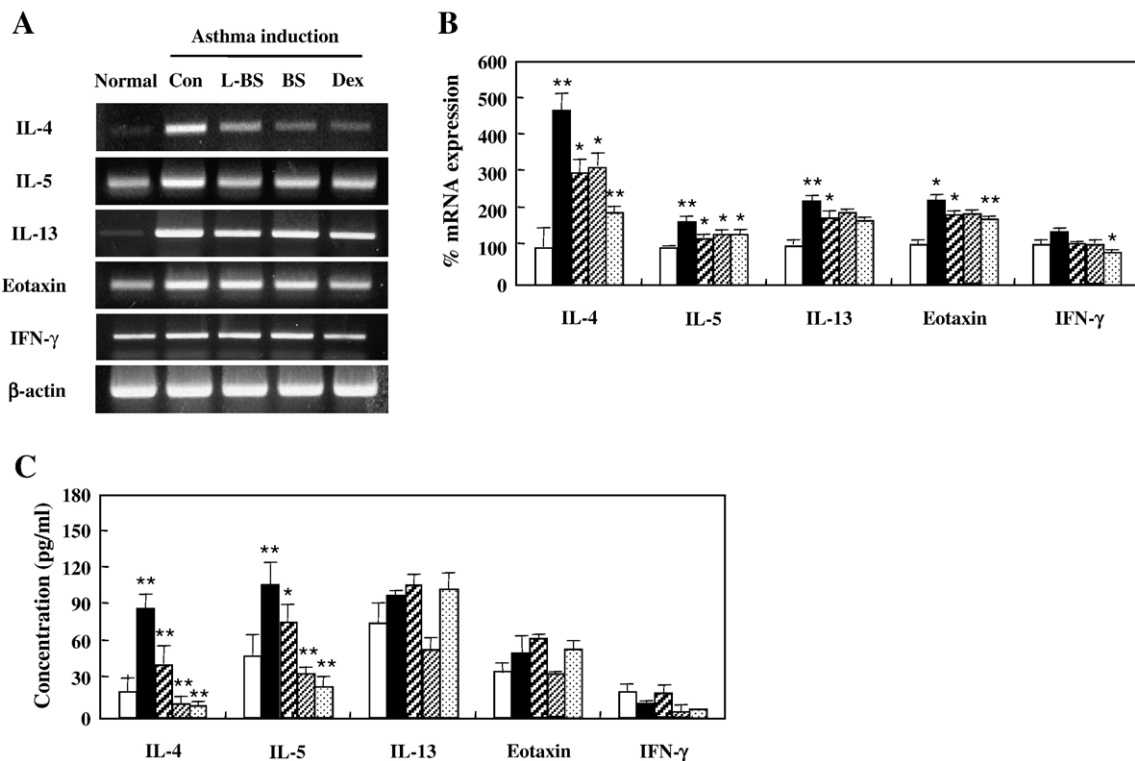


Fig. 5. Effect of L-BS and BS on the expression of IL-4, IL-5, IL-13, IFN- γ and eotaxin in lung tissues and BAL fluid. (A) Lung tissues were excised from ovalbumin-induced mice untreated (Con; closed bar) or treated with 20 μ g of lactose- β -sitosterol (L-BS; thick hatched bar), β -sitosterol (BS; thin hatched bar) or dexamethasone (Dex; dotted bar) between the sensitization and inhalation period. The negative control group (Normal; open bar) was sensitized and challenged with PBS without drug administration. The total RNA was extracted from the tissues. The mRNA levels of IL-4, IL-5, IL-13, eotaxin and IFN- γ were analyzed by RT-PCR as described in the Methods section. The bands were normalized with β -actin. The data are expressed as representative of five independent experiments. (B) The densitometric data are expressed as the means $\pm$ S.E.M. of five independent experiments, in relation to the negative group, which was set at 100%. (C) BAL fluid was collected from ovalbumin-induced mice untreated (Con; closed bar) or treated with 20 μ g of lactose- β -sitosterol (L-BS; thick hatched bar), β -sitosterol (BS; thin hatched bar) or dexamethasone (Dex; dotted bar) between the sensitization and inhalation period. The negative control group (Normal; open bar) was sensitized and challenged with PBS without drug administration. The protein expression of IL-4, IL-5, IL-13, eotaxin and IFN- γ in BAL fluid was analyzed by ELISA as described in the Methods section. The data are expressed as the means $\pm$ S.E.M. of five independent experiments. * p < 0.05 and ** p < 0.01 are assessed as a significant difference between the normal group and asthma control group or between the asthma control group and drug-treated group.

4. Discussion

Asthma is a chronic inflammation in lung and the number of people suffering from asthma has been increasing around world for the last twenty years. A variety of drugs, including steroids, leukotriene inhibitors, mast cell stabilizers, and β_2 adrenergic agonists, have been administered to treat asthma, and the majority of them fully satisfy the therapeutic effect we hope to obtain. Therefore, for the first time, we investigated the possibility of L-BS and BS as new therapeutic drugs for the treatment of allergic asthma using an asthma-induced mouse model. L-BS significantly inhibited the leucocytosis and eosinophilia, and BS exhibited a similar effect. L-BS and BS blocked the elevated mRNA and protein expression of IL-4 and IL-5 in spite of differing degrees of inhibition and alleviated the asthmatic reaction, including the airway inflammation and mucus secretion in the lung. L-BS and BS inhibited the viability of the splenocytes of the normal and asthmatic mice. L-BS exhibited more potent effects than BS.

β -sitosterol (BS) and BS glucoside are found in a variety of plants and are similar to the structure of cholesterol. The possible use of β -sitosterol (BS) and BS glucoside as therapeutic drugs in immune-mediated

disorders has been reported [10]. Although the effect of BS or BS and BS glucoside was elucidated by a few in vitro studies, the sole effect of BS glucoside has not yet been fully characterized. We first synthesized L-BS as a kind of BS glucoside from BS using a new method and investigated the suppressive and comparative effects of L-BS and BS on allergic inflammation in a mouse model of asthma.

The migration of inflammatory cells, specifically eosinophils and lymphocytes, into the lung is the primary contributor to the development of allergic airway inflammation [3]. Our results demonstrated that L-BS totally diminished the elevated inflammatory cells and absolutely reduced the eosinophilia and lymphocytosis in the BAL fluid after asthma induction (Fig. 3A–C). BS weakly decreased the cells in the BAL fluid in asthmatic mice in comparison to L-BS. Infiltrated eosinophils and other leukocytes released ROS, which resulted in severe pathogenesis via the activation of inflammatory cells, mucus secretion and the contraction of smooth muscle [13]. Both L-BS and BS lessened ROS production (Fig. 3D). We used a histochemical method to examine the lung tissue and found marked eosinophil infiltration in the asthmatic mice (Fig. 4A and C). The inflammation associated with the eosinophilia significantly decreased

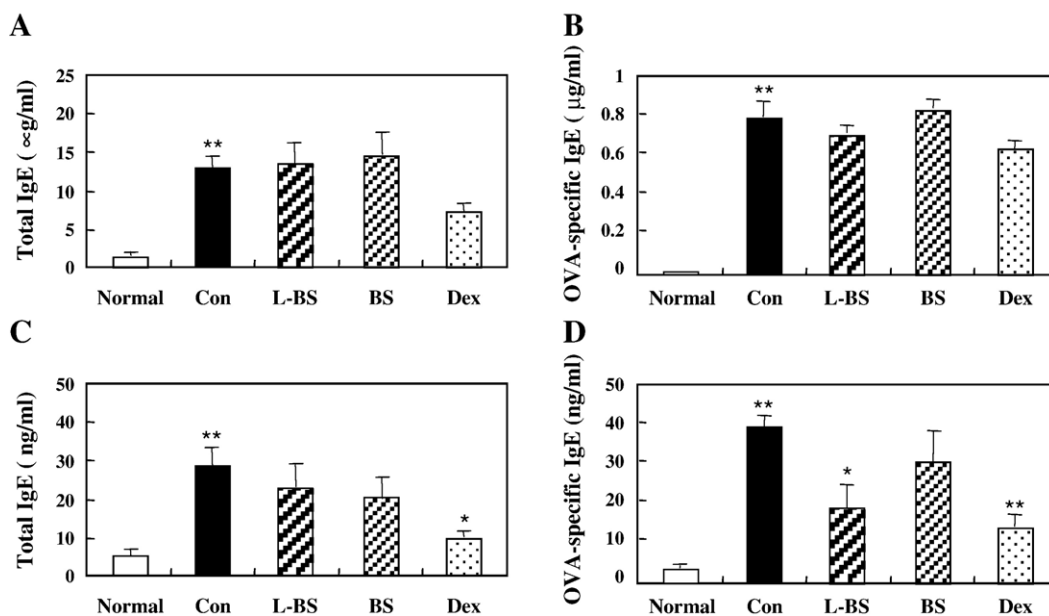


Fig. 6. Effect of L-BS and BS on the total IgE and ovalbumin-specific IgE levels in the serum and BAL fluid. Serum and BAL fluid were collected from ovalbumin-induced mice untreated (Con; closed bar) or treated with 20 μ g of lactose- β -sitosterol (L-BS; thick hatched bar), β -sitosterol (BS; thin hatched bar) or dexamethasone (Dex; dotted bar) between the sensitization and inhalation period. The negative control group (Normal; open bar) was sensitized and challenged with PBS without drug administration. The total IgE and ovalbumin (OVA)-specific IgE levels in the serum (A, B) and BAL fluid (C, D) were analyzed by ELISA as described in the Materials and methods section. The data are expressed as the means $\pm$ S.E.M. of five independent experiments. * $p < 0.05$ and ** $p < 0.01$ are assessed as a significant difference between the normal group and asthma control group or between the asthma control group and drug-treated group.

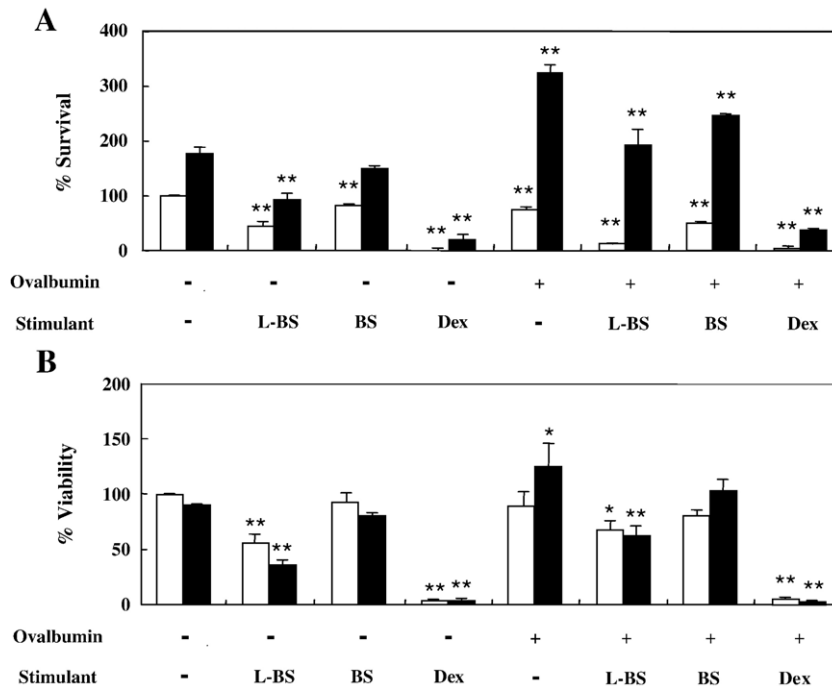


Fig. 7. Effect of L-BS and BS on the survival rate of the splenocytes. The splenocytes were isolated from the spleens of normal (open bar) and asthmatic control mice (closed bar). 2×10^5 splenocytes in 100 μ l of DMEM medium containing 10% FBS were seeded onto a 96-well culture plate. L-BS, BS or dexamethasone (1 μ g/ml) in the absence or presence of 100 μ g/ml ovalbumin was added to the individual well and then the plate was incubated for 48 h at 37 $^{\circ}$ C in a CO₂ incubator. For MTT assay (A), the cells were incubated with 10 μ l of MTT solution for 4 h and then 100 μ l of solubilization solution was added to the each well. After 24 h incubation, the absorbance was measured by using an ELISA reader at 550 nm. For the apoptosis assay (B), the cells were incubated with annexin V-FITC and PI and analyzed by FACSsort cytofluorimeter using CellQuest software. Negative cells against annexin V and PI staining were considered as viable or non-apoptotic cells. The data are expressed as the means $\pm$ S.E.M. of three independent experiments, in relation to the negative group, which was set at 100%. * p < 0.05 and ** p < 0.01 are assessed as a significant difference between the untreated group and drug-treated group or between ovalbumin-treated group and ovalbumin plus drug-treated group.

after L-BS treatment and weakly diminished after BS treatment, which is in agreement with the data from Fig. 3. Mucus hypersecretion as well as inflammation has been recently understood to be a cause of airway obstruction and an important clinical feature in asthma management and prevention [14]. L-BS also ameliorated the increased mucus production by goblet cell hyperplasia, indicating a distinguishing mark of airway remodeling (Fig. 4B and C).

Asthma displays elevated levels of Th2 cytokines, IL-4, IL-5 and IL-13. Th2 cytokines are secreted by resident cells such as epithelial cells, macrophages, and mast cells as well as infiltrated cells such as eosinophils and lymphocytes [15]. IL-4 and IL-13 play important roles in IgE switching in B cells, the development of eosinophil infiltration into the airways, and mucus hypersecretion [16]. IL-5 is an essential molecule for the activation and survival of eosinophils and airway hyperresponsiveness [16]. These results have been demonstrated by a number of reports using transgenic

or knockout mice and by blocking antibodies against cytokines [17–21]. Our results showed that L-BS and BS inhibited the increased mRNA and protein expression of IL-4 and IL-5 (Fig. 5). BS exhibited more effective inhibitory effects on IL-4 and IL-5 expression than L-BS, in contrast to the superior effect of L-BS on the inhibition of the eosinophil number and the appearances of lung tissue (Figs. 3 and 4). These conflicting results cannot be presently explained. However, these results are possible and may be important to the understanding that asthma is a complex disease elicited by a variety of molecular and cellular factors in lung tissue and the total body. The protein expression of IL-13 and eotaxin, a potent chemoattractant for eosinophils, is generally elevated after asthma induction. The expression of both proteins increased in this experiment, but not significantly (Fig. 5C). These results may be affected by a different assay system and protocol on asthma induction in comparison with other reports. The decreased expression of the IFN- γ protein in the asthma control was weakly

increased by L-BS (Fig. 5C). The specific mechanism underlying the balance of the Th1 and Th2 cytokines or regulation of chemokine expression of L-BS and BS is currently under investigation.

IgE is associated with the early phase of allergic asthma. After the binding of the allergen to IgE, the complex interacts with the IgE receptor and activates mast cells to secrete numerous mediators, which causes the disease to get worse [22]. A recent report suggested the importance of local allergen-specific IgE within the bronchi, which is unreliable to a concentration of serum IgE [23]. As shown in Fig. 6, the total IgE level in the serum and BAL fluid increased after asthma induction and was not altered by L-BS or BS. However, the concentration of ovalbumin-specific IgE in the BAL fluid was significantly blocked by L-BS. These results indicate that L-BS diminished the selectively of the local IgE level without altering the total circulating IgE. BS also blocked ovalbumin-specific IgE in the BAL fluid, but not significantly. The superior effect of L-BS on the inhibition of the IgE level is constant with the results from the histological response, including inflammation and mucus generation. In addition, L-BS reduced the survival rate of the splenocytes (Fig. 7) and mast cell migration in response to stem cell factor (data not shown). In spite of the results presented in this study, we still do not know how L-BS and BS attenuate the airway inflammation associated with asthma; therefore, we intend to elucidate the exact mechanism underlying the anti-asthmatic function of L-BS and BS in future studies.

Corticosteroids have been known as one of the most attractive anti-inflammatory drugs for the therapy of a wide variety of allergic diseases. Clinical and review studies demonstrated that the treatment of asthma with corticosteroids inhibits the infiltration of eosinophils and lymphocytes in the bronchial mucosa and diminishes Th2 cytokine expression [24,25]. Dexamethasone blocked eosinophil recruitment into lung, and Th2 cytokine and IgE expression (Figs. 3–6), which is consistent with the findings of previous studies. L-BS exhibits a similar anti-asthmatic effect despite the differing degree of inhibitory effects. We examined the weight of each mouse and lung to evaluate the health of each mouse after drug treatment. L-BS (19.9 ± 1.1 g, 0.23 ± 0.06 g) and BS (19.7 ± 0.2 g, 0.23 ± 0.03 g) treatment did not alter the weight of the mouse or the lung; however, dexamethasone (18.2 ± 0.8 g, 0.18 ± 0.04 g) significantly decreased in comparison to the normal (19.0 ± 1.1 g, 0.22 ± 0.06 g) and asthma control levels (19.8 ± 0.7 g, 0.23 ± 0.05 g) ($p < 0.05$). These results may indicate the possibility of the non-toxicity of L-BS and BS and the possibility of an increased effect by

treatment with concentrations higher than 1 mg/kg. In addition, we intend to further investigate the optimal dosage, constitution and method of L-BS and BS administration.

In summary, our results strongly indicate that L-BS and BS suppressed the expression of cytokines and IgE, as well as the recruitment of eosinophils, and these results diminish pathological injury of lung. This study may support the possibility of L-BS and BS as effective compounds for the treatment of asthma and as possible targets for the development of new therapeutic drugs in allergic diseases in addition to asthma.

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