

Silkworm dropping extract regulates food allergy symptoms via inhibition of Th2-related responses in an ovalbumin-induced food allergy model

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Abstract

BACKGROUND: Silkworm droppings have long been used in traditional medicine to remedy allergic itching, palsy, blood circulation problems, and arthritis in Asian countries. To investigate the anti-allergic effect of silkworm dropping extract (SDE) and its mechanism, we used a mouse model of food allergy induced by ovalbumin (OVA).

RESULTS: SDE ameliorated the symptoms of OVA-induced food allergies, and the levels of T helper 2 (Th2)-related cytokines [such as interleukin (IL)-4, IL-5, IL-10, and IL-13] were found to be significantly decreased in both the spleen and mesenteric lymph nodes by SDE. Furthermore, SDE treatment directly inhibited OVA permeation, IL-4 production, and degranulation of mast cells; in contrast, immunoglobulin E (IgE) production from B cells was not affected.

CONCLUSION: These results suggest that SDE has potential anti-allergic activities, and SDE may be useful in the treatment/prevention of allergic disorders such as food allergies, serving as therapeutic agents.

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Keywords: silkworm dropping; food allergy; immunoglobulin E; Th2-related cytokines

INTRODUCTION

Well-known allergic disorders, such as atopic dermatitis, allergic asthma, allergic rhinitis, allergic contact dermatitis, and food allergy, present a considerable and evolving social health problem.^{1–3} The patients suffer from various allergic symptoms such as anaphylaxis, itching, and dyspnea. In particular, food allergy describes an immunological response caused by food ingredients (e.g. milk, soy, peanut, seafood, and egg).^{4,5} The prevalence of food allergies is growing in both the adult and child population.^{6,7} It usually occurs with severe symptoms including anaphylaxis, diarrhea, vomiting, erythema, urticaria, and eczematous rash.⁸ It was reported that these symptoms were caused by food specific immunoglobulin E (IgE), which plays an essential role in food allergic responses.^{9,10} Briefly, allergens (antigens) penetrate into the gastrointestinal tract and are then presented by the major histocompatibility complex (MHC) class II molecules expressed on the surface of antigen presenting cells (APCs). Both allergen and MHC class II molecules stimulate and activate naïve CD4 T cells. After activation, the cells differentiate into allergen-specific T helper 2 (Th2) cells that can produce cytokines, such as interleukin (IL)-4, IL-5, IL-10, and IL-13.¹¹ Th2-mediated cytokines can promote IgE production from B cells, and the IgEs bind to a high-affinity receptor (FcεRI) on the surface of mast cells. This state is called an atopic state, and the mast cells degranulate after crosslinked

binding to the newly permeated allergens, resulting in secretion of allergic inflammatory mediators such as histamine, proteases, leukotrienes, and cytokines.^{2,10} These mediators can lead to an anaphylactic response in individuals with a food allergy. To treat allergic disorders such as food allergies, steroids, anti-histamine, and immune suppressors called anti-allergic medicines were developed and are currently in use. However, their long-term use may cause side effects such as osteoporosis.^{12,13} Given these potential risks, several studies propose the use of natural substances, which have the potential to treat allergic diseases without side effects.

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A Korean traditional medical book called *Dongui Bogam* introduced the beneficial effects of various natural products for treatment of inflammatory and allergic diseases.¹⁴ Among these natural substances, silkworm dropping (SD), which is known as Jam-bun, was reported to strengthen internal organs, protect against diabetes, and treat diabetes. Furthermore, it is widely used to treat palsy, blood circulation problems, fever, unhealthy eyes, headache, itching, and arthritis in Asian countries.^{15,16} Recently, it was reported that SD treatment suppressed the development of atopic dermatitis by decreasing production of several cytokines such as IL-4 and also by down-regulating the mRNA expression of IL-6, MCP-1, and MIP-1 α cytokines.¹⁵ This report provided scientific evidence of the anti-inflammatory effects of SD. However, the anti-allergic effects of SD have not yet been clearly established. Therefore, we tried to determine the anti-allergic effects of SDs in a mouse model of food allergy.

MATERIALS AND METHODS

Preparation of silkworm dropping extract

Silkworm dropping extract (SDE) was supplied by Health Recovery Food Co., Ltd (Jangsu County, Korea). Briefly, SDs obtained from fifth instar silkworms were dried under shade and immersed in water for 15 days to eliminate toxicity. After completely drying, SDs (20 kg) were mixed with ethanol (100 L) and extracted by ultrasonic waves at 20 °C for 3 days. The supernatant from the extracted solution was collected by centrifugation, and the solvent was evaporated using a rotary evaporator. The concentrated extract was dried in a freeze dryer and stored at 4 °C until use. The SDE was standardized to contain 0.16 mg g⁻¹ (milligrams of pheophorbide-a per gram of SDE) (content range of $\pm 20\%$, 0.16 mg $\pm$ 0.032 mg g⁻¹) of pheophorbide-a.

Animals

Five-week-old Balb/c female mice were purchased from Orient-Bio Inc. (Kyeonggi, Korea). The mice were kept under specific pathogen-free conditions (23 $\pm$ 2 °C, 12 h light/dark cycles). Food and water were provided *ad libitum*. All animals were maintained in accordance with the Korea Food Research Institute guidelines for animal use and care (permit number: KFRI-M-15026).

Induction of food allergy

Induction of food allergy was performed as previously described.¹⁷ Briefly, mice were randomly divided into four groups ($n = 6$): naïve (saline, no induced food allergy), sham (saline, induced food allergy), SDE (200 mg kg⁻¹, induced food allergy) and dexamethasone (2.5 mg kg⁻¹, induced food allergy). Naïve and sham groups were orally administered saline. Each SDE or dexamethasone group was given SDE (200 mg kg⁻¹) or dexamethasone (2.5 mg kg⁻¹), respectively. Oral administration was given from day 14 to 43. For induction of an experimental food allergy, the mice were immunized with 20 μ g ovalbumin (OVA) in the presence of alum (2 mg) by intraperitoneal injection on day 0 and 14. Beginning on day 28, the mice received OVA (50 mg head⁻¹) in saline by oral gavage every 3 days in total six times (Fig. 1). Food allergy symptoms were monitored by examining the rectal temperature and anaphylaxis response during the 60 min following OVA administration. The rectal temperature was measured every 20 min for 1 h by Electronic Thermometers (Physitemp Instruments, Inc, Clifton, NJ, USA). Anaphylactic response was assessed by standard criteria for up to 1 h. The anaphylaxis score was determined using a

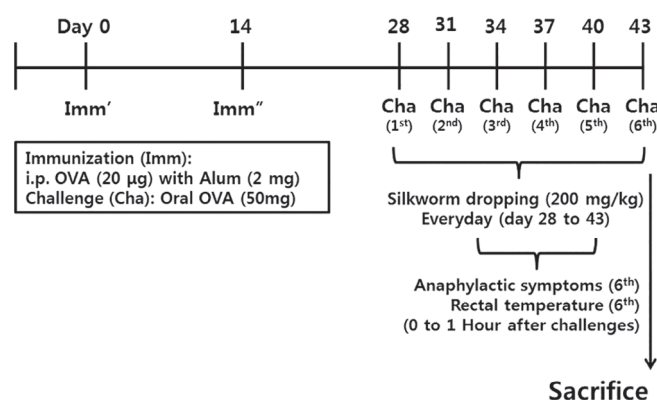


Figure 1. Experimental schedule of ovalbumin (OVA)-induced food allergy in a mouse model. Female Balb/c mice (5-weeks old) were divided into naïve ($n = 6$), sham ($n = 6$), silkworm dropping extract (SDE) ($n = 6$), and dexamethasone ($n = 6$) groups. To induce an allergic response, mice were immunized twice with 20 μ g OVA adsorbed in 2 mg mL⁻¹ alum by intraperitoneal injection on day 0 and 14. Starting on day 28, the immunized mice were orally challenged with 50 mg OVA in saline every 3 days six times in total. SDE [200 mg kg⁻¹ body weight (BW)] or dexamethasone (2.5 mg kg⁻¹ BW) was orally administered daily from day 28 to day 43. Anaphylaxis and rectal temperature were measured as indicators of food allergy symptoms.

scoring system as described.¹⁸ The criteria of anaphylaxis response were described as: 0, no symptoms; 1, reduced activity, trembling of limbs; 2, loss of consciousness, no activity upon prodding; 3, convulsions; 4, death. Scoring was conducted by three independent monitors, and the results were represented as an average for each group.

Measurement of total and OVA-specific IgE in sera

Blood samples were collected from the orbital venous plexus on day 43. The serum samples were separated by centrifugation at 10000 $\times$ g for 15 min at 4 °C. The concentration of total and OVA-specific IgE in the mouse serum was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols (BD Pharmingen, San Diego, CA, USA).

Determination of cytokine production in the spleen and mesenteric lymph node of mice

To measure cytokine levels, the spleen and mesenteric lymph node (mLN) were removed from each mouse after sacrifice. Both tissues were homogenized and suspended into single cells. Splenocytes were lysed using red blood cell lysis buffer (Sigma-Aldrich, St Louis, MO, USA) for removing red blood cells. The prepared cells (5 000 000 cells well⁻¹) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) under 5% carbon dioxide (CO₂) at 37 °C. After 72 h, the supernatants were collected, and then the levels of the cytokine [IL-4, IL-5, IL-10, IL-12, IL-13, and interferon-gamma (IFN- γ)] were evaluated by ELISA according to the manufacturer's instructions (BD Pharmingen).

Allergen permeation in human intestinal epithelial Caco-2 cell

Human intestinal epithelial Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1000 mg L⁻¹ of glucose and supplemented with 10% FBS, 1% non-essential amino acid (NEAA), 100 U mL⁻¹ of penicillin, and 100 μ g mL⁻¹ of streptomycin at 37 °C with an atmosphere of 5% CO₂. Caco-2 cells

were seeded at a density of $200\,000\text{ cells mL}^{-1}$ in a 12-transwell plate (Costar, Corning, NY, USA) for 3 weeks. Determination of the allergen permeation was described in a previous study.¹⁹ Briefly, Caco-2 cells were treated with SDE ($100\text{ }\mu\text{g mL}^{-1}$) and bile salts ($160\text{ }\mu\text{g mL}^{-1}$) for 60 min, and then OVA was added for 3 h at $37\text{ }^{\circ}\text{C}$. The trans-epithelial electrical resistance (TEER) value was measured as the intestinal barrier function, and OVA influx was detected as the amount of allergen permeation by ELISA.

Measurement of Th2-associated immune response in splenocytes

Balb/c mice (5-weeks old, female) were intraperitoneally sensitized with OVA ($20\text{ }\mu\text{g}$; Sigma-Aldrich) mixed alum (2 mg ; Pierce, Rockford, IL, USA) on day 0 and 14. After 2 weeks, the mice were sacrificed, and the isolated spleen was suspended to single cells for the culture of splenocytes. The single cells were seeded into 96-well plates ($1\,000\,000\text{ cells well}^{-1}$) in RPMI 1640 medium supplemented with 10% FBS. The splenocytes were then cultured with SDE ($1\text{--}30\text{ }\mu\text{g mL}^{-1}$) in the absence or presence of OVA ($100\text{ }\mu\text{g mL}^{-1}$) for 72 h. The medium was collected, and the level of IL-4 cytokine was detected by ELISA according to the manufacturer's instructions (BD Pharmingen).

IgE production in primary B cells from sensitized mice

Purified primary B cells were isolated from the spleen of sensitized mice using MagniSort® Mouse CD45R (B220) Positive Selection Kit (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. For detection of IgE production, purified CD45R⁺ B cells were cultured with lipopolysaccharide ($10\text{ }\mu\text{g mL}^{-1}$), IL-4 (5 ng mL^{-1}) and SDE ($1\text{--}30\text{ }\mu\text{g mL}^{-1}$) for 72 h. The supernatants were then collected, and IgE was measured by ELISA (BD Biosciences, San Diego, CA, USA).

Degranulation in rat peritoneal mast cell

Rat peritoneal mast cells (RPMCs) were isolated as previously described.²⁰ Purified RPMCs ($1\,000\,000\text{ cells mL}^{-1}$) were resuspended in HEPES-Tyrod buffer. The RPMCs were pretreated with SDE ($0\text{--}100\text{ }\mu\text{g mL}^{-1}$) for 10 min at $37\text{ }^{\circ}\text{C}$ and observed within 10 min of addition of compound 48/80 ($5\text{ }\mu\text{g mL}^{-1}$) under phase contrast microscopy and photographed as described.²¹ The mast cells ($\times 1000$) were classified as follows: (i) extensively degranulated ($> 50\%$ of the cytoplasmic granules exhibiting fusion, staining alterations, and extrusion from the cell), (ii) slight to moderately degranulated ($10\text{--}50\%$ of the granules exhibiting fusion or discharge), or (iii) normal.

Statistical analysis

Each result is expressed as the mean $\pm$ standard deviation. Differences between the experimental data were assessed by one-way analysis of variance (ANOVA) followed by F-protected Fisher's least significant difference test.

RESULTS

SDE alleviates food allergy symptoms in an OVA-induced food allergy mouse model

We investigated whether SDE ameliorates the symptoms of food allergy in a mouse model. Food allergy symptoms, such as anaphylactic response and change in rectal temperature, were evaluated during 60 min after the sixth challenge with OVA. As a result,

the sham group showed a significant decrease in rectal temperature compared to that of the naïve group (sham versus naïve, -8.8 ± 1.8 versus 0.5 ± 0.6) (Fig. 2(A)). Furthermore, anaphylactic response in the sham group was 2.33 points higher in the group challenged with OVA compared to that in the naïve group (Fig. 2(B)). In contrast, administration of SDE attenuated the rectal temperature and anaphylactic response induced by OVA by $-4.1\text{ }^{\circ}\text{C}$ and 1.37 points, respectively, compared to that in the sham group. These results demonstrate that administration of SDE can attenuate OVA-induced food allergy symptoms in a mouse.

Administration of SDE inhibits total and OVA-specific IgE serum levels

It is known that food allergy responses, including anaphylactic shock, depend on IgE levels. The best known functions of IgE are in instigating classic immediate hypersensitivity reactions, whereupon exposure to the offending allergen elicits allergic symptoms in only minutes.²¹ Thus, we investigated whether administration of SDE suppresses the increase in IgE levels caused by OVA in a mouse model of food allergy. The sham group exhibited the highest levels of total ($9671 \pm 2593\text{ ng mL}^{-1}$) and OVA-specific IgE (OD (optical density) 0.549 ± 0.036), whereas the total and OVA-specific IgE levels in the group administered SDE were significantly reduced compared with the levels in the sham group ($5725 \pm 1715\text{ ng mL}^{-1}$ and

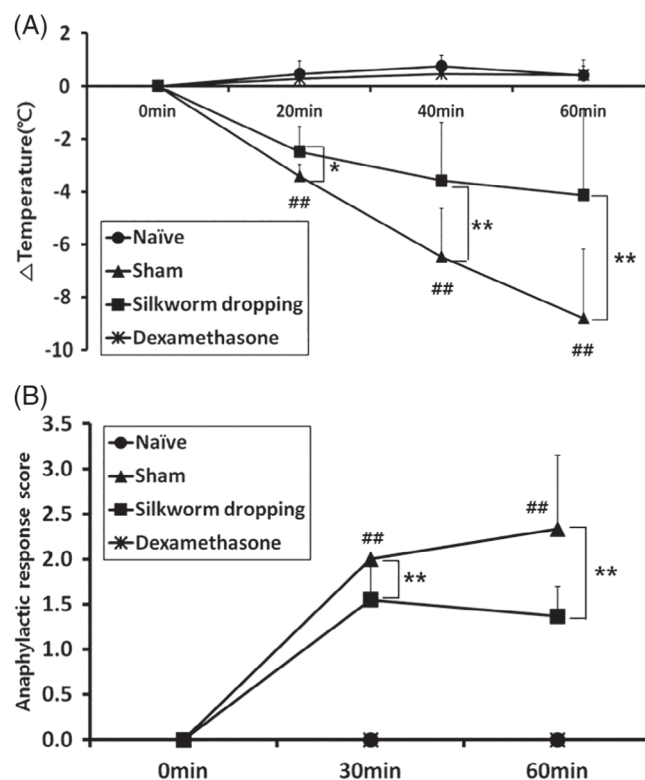


Figure 2. Effect of silkworm dropping extract (SDE) on ovalbumin (OVA)-induced food allergy symptoms. Food allergy symptoms induced by OVA were evaluated (decreased rectal temperature and anaphylactic response) for 1 h after challenge with OVA. (A). Rectal temperature was measured every 20 min for 1 h after the sixth challenge with OVA. (B). Anaphylactic response score was evaluated every 30 min for 1 h after the sixth challenge with OVA. Each value is presented as the mean $\pm$ standard deviation. Bars represent a significant difference from the naïve group at $^{##}P < 0.01$ or the sham group at $^{*}P < 0.05$ and $^{**}P < 0.01$. Data were analyzed using analysis of variance followed by F-protected Fisher's least significant difference test.

OD 0.408 ± 0.055) (Table 1). In the present study, we confirmed the correlation between food allergy symptoms and IgE levels and showed that administration of SDE has potential for the alleviation of food allergy responses.

SDE inhibits Th2-mediated immune responses in both the systemic and gut immune system

Various allergic diseases, including food allergy, are caused by dominant Th2-mediated immune responses. We investigated the levels of Th2-related cytokines in a mouse model of food allergy. As a result, IL-4, IL-5, IL-10, and IL-13, which are well-known Th2-related cytokines, were strongly enhanced after administration of OVA in the sham group. However, administration of SDE dramatically inhibited Th2-related cytokines (IL-4, IL-5, IL-10, and IL-13) (Fig. 3(A–D)). Since Th2-mediated immune responses are known to be regulated by Th1-mediated immune responses,²² we investigated whether SDE suppresses Th2-mediated immune responses through either enhancement of Th1-mediated immune responses or specific inhibition of the Th2-mediated immune response. We found that the levels of IL-12 and IFN- γ , well-known Th1-related cytokines, were not changed by SDE treatment (data not shown). Therefore, the administration of SDE specifically suppressed Th2-mediated immune responses without affecting the Th1-mediated immune responses.

We also investigated the effect of SD in gut immune responses, because SDs were orally digested. In mLN isolated from mice, Th2- and Th1-related cytokines were measured by ELISA. As a result, the administration of SDE also inhibited the production of Th2-associated cytokines in the gut immune system but did not inhibit Th1-associated cytokines (Fig. 3(E–H)). These results demonstrated that the administration of SD attenuated food allergy symptoms by specifically inhibiting Th2-mediated immune responses in both the systemic and gut immune systems.

SDE directly regulates allergen permeation, Th2-mediated immune responses, and degranulation of mast cells on continuous allergic responses

We investigated how SDs regulate continuous allergic immune responses. We targeted the following four mechanisms of allergic responses: allergen (OVA) permeation, Th2-mediated immune responses, IgE production from B cells, and degranulation of mast cells. We then examined whether SDE directly regulates each pathway. As a result, we showed that SDE treatment directly inhibited OVA permeation across intestinal epithelial cells through enhancement of the barrier function, and the treatment directly suppressed IL-4 production (main Th2-related cytokine) (Fig. 4). Furthermore, compound 48/80-induced degranulation of mast cells was prevented by SDE. In contrast, IgE production in B cells was not affected by SDE treatment. Therefore, we suggest that SDE is able to attenuate food allergic responses through the negative regulation of allergen permeation, Th2 immune responses, and degranulation of mast cells.

DISCUSSION

SDs are excreted from *Bombyx mori* grown on mulberry. The *Bombyx mori* digests only 40% of protein in mulberry and excretes the remainder.²³ Mulberry contains various ingredients that were shown to have various physiological effects, including anti-oxidative and anti-hyperlipidemic effects, whereas SD contains both the mulberry ingredients and metabolites synthesized by enzymes and microbes in the intestine of the silkworm. However, the physiological effects of SD compared to that of mulberry leaves were not characterized well previously. In our present study, we investigated how SDE regulates continuous allergic immune responses. Four potential target sites were examined to determine the regulatory mechanism of SDE using various cells such as intestinal epithelial cells, Th2-dominant splenocytes, CD45⁺ B cells, and RPMCs. We showed that SDE directly inhibited allergen permeation, Th2-allergic responses, and degranulation of mast cells. However, SDE did not suppress IgE production of B cells induced by Lipopolysaccharides (LPS) and IL-4. Interestingly, our results showed that the oral administration of SDE reduced serum IgE levels in a food allergy model. We thought that the administration of SDE already regulated initiative phase on continuous allergic responses such as OVA permeation across intestinal epithelial cells, IL-4 secretion from Th2 cells, and then IgE production might be reduced by the regulatory effects of SDE in the early phase of food allergy. This may be because IL-4 generally plays essential roles in the induction of immunoglobulin class switching to IgE and clonal expansion of naive and IgE⁺ memory B-cell populations.²⁴

In this study, we suggested one anti-allergic mechanism in that the SDE enhanced intestinal barrier function and suppressed the OVA penetration across intestinal epithelial cells and then it might be connected with down-regulation of the Th2-related immune response. The intestinal epithelium was classified as an innate immune system, that plays the role of oral tolerance by selectively permeating nutrients, antigens, toxins and water. Food allergy was associated with defect of the intestinal barrier function, and antigen penetrated across the dysfunctional intestinal barrier leading to recruitment of mast cells, production of allergen specific-IgE, and secretion of Th2-related cytokines, eventually causing the food allergy. It was reported that permeability of the intestinal barrier was correlated with food allergy symptoms severity.²⁵ Moreover, enhancement of the intestinal barrier function is important to prevent inflammatory and allergic diseases such as inflammatory bowel disease, food allergy, and leaky gut syndrome.^{26,27} Our results showed that SDE increased TEER value as an index of the intestinal barrier function and reduced the permeation of OVA as an allergen in Caco-2 cell monolayers. We previously showed that *Scutellaria baicalensis* and its active compound baicalein inhibited allergen permeation through enhancement of the intestinal barrier.^{17,18,28} This could function to dramatically attenuate food allergy symptoms. Therefore, we suggest that the effect of SDE

Table 1. Serum immunoglobulin E (IgE) levels in a mouse model of food allergy

	Naïve	Sham	Silkworm dropping	Dexamethasone
IgE (ng ml ⁻¹)	553.5 ± 366.6	9671.1 ± 2593.5 ^{##}	5725.9 ± 1715.0 [*]	5775.3 ± 2532.7 [*]
Ovalbumin-specific IgE (OD 450 nm)	0.057 ± 0.003	0.536 ± 0.047 ^{##}	0.408 ± 0.056 ^{**}	0.556 ± 0.152

Each value is presented as the mean ± standard deviation. A significant difference from the naïve group at ^{##}*P* < 0.01 or the sham group at ^{*}*P* < 0.05 and ^{**}*P* < 0.01 is shown. Data were analyzed using analysis of variance followed by F-protected Fisher's least significant difference test.

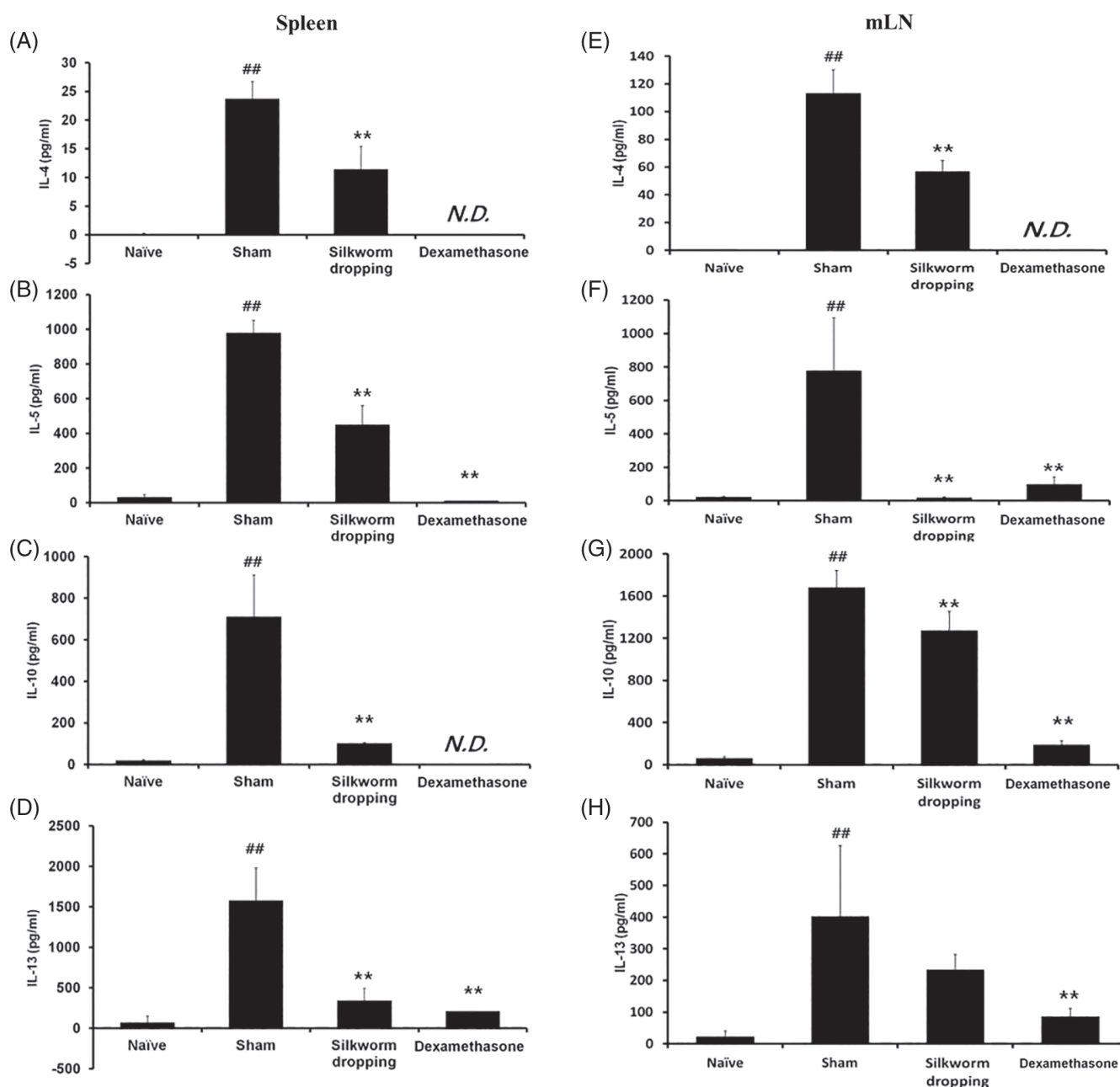


Figure 3. Anti-allergic effects of silkworm dropping extract (SDE) on the production of T helper 2 (Th2)-associated cytokines in splenocytes and mesenteric lymph node (mLN). Mice were sacrificed by cervical dislocation on day 43, and their spleens and mLNs were removed. Splenocytes were isolated from the spleen and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) for 72 h in the presence of ovalbumin (OVA). Cytokines produced from splenocytes were detected by ELISA. Interleukin (IL)-4 (A), IL-5 (B), IL-10 (C), and IL-13 (D) were measured as Th2-related cytokines. Furthermore, lymphocytes isolated from mLN cultured in the presence of OVA for 72 h. Cytokines secreted from mLN lymphocytes were analyzed by ELISA. Levels of IL-4 (E), IL-5 (F), IL-10 (G), and IL-13 (H) were measured. Each value is presented as the mean $\pm$ standard deviation ($n = 3$). Bars represent a significant difference for the naïve group at $^{##}P < 0.01$ or the sham group at $^{**}P < 0.01$. Data were analyzed using analysis of variance followed by F-protected Fisher's least significant difference test. N.D. indicates 'Not Detected.'

on the intestinal barrier contributes to its amelioration of food allergies.

Furthermore, as another anti-allergic mechanism, the SDE inhibited Th2-associated cytokines such as IL-4, IL-5, IL-10, and IL-13. Inhibitory mechanisms of SDE for Th2-related cytokines can be presumed by three hypotheses. The first, suppression of OVA penetration across intestinal epithelial cells might be connected with down-regulation of the Th2-related immune response. The second, the SDE directly inhibited Th2-mediated

immune responses. The third, the SDE induced regulatory T (Treg) cells, resulting in inhibition of Th2-mediated immune responses. Among these hypotheses, we suggest that the SDE directly inhibited Th2-mediated immune responses. Since, in a previous study, the SDE alleviated the symptoms of trimellitic anhydride (TMA)-induced allergic contact dermatitis by inhibiting Th2-related immune responses, and the mouse model was induced by topical (ears) treatment of TMA,²⁹ we therefore investigated whether the SDE induces Treg cells. As a result, the

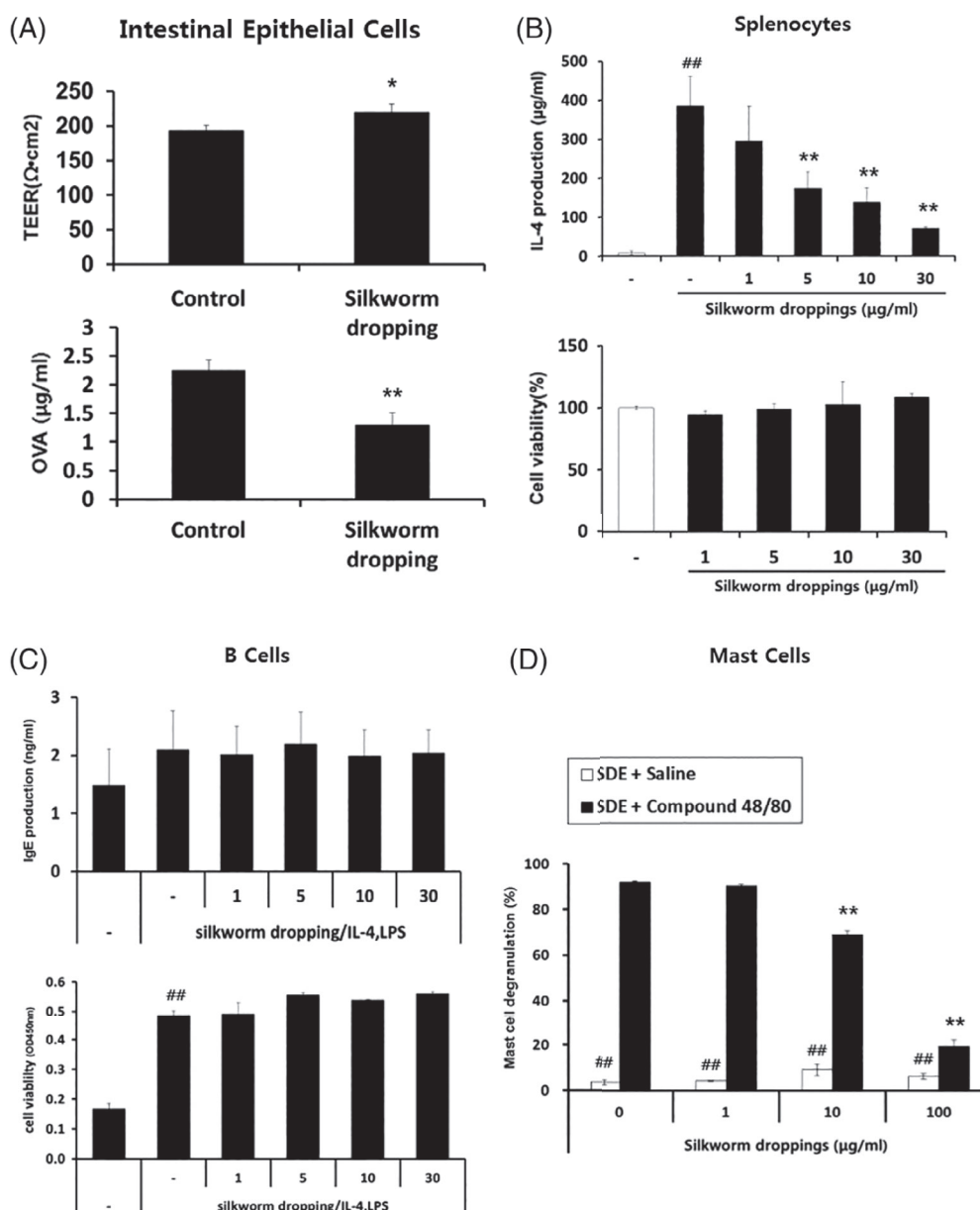


Figure 4. Anti-allergic mechanisms of silkworm dropping extract (SDE) on allergic responses. To determine the anti-allergic mechanism of SDE on continuously occurring allergic responses, we focused on allergen permeation into intestinal epithelial cells, interleukin (IL)-4 production from splenocytes, immunoglobulin E (IgE) production from B cells (CD45R⁺ cells), and degranulation of mast cells. (A) Caco-2 cell monolayers were used as intestinal epithelial cells, and the cells were treated with 100 $\mu\text{g mL}^{-1}$ SDE and 400 $\mu\text{g mL}^{-1}$ ovalbumin (OVA) for 3 h. (B) Splenocytes isolated from an OVA-immunized mouse were stimulated with OVA, and the cells were treated with 1–30 $\mu\text{g mL}^{-1}$ of SDE for 72 h. The production of IL-4 from the supernatants was measured by ELISA. (C) CD45R⁺ cells were isolated from mice using MACS system and were stimulated with LPS (10 $\mu\text{g mL}^{-1}$) and IL-4 (5 $\mu\text{g mL}^{-1}$), and the cells were treated with 1–30 $\mu\text{g mL}^{-1}$ of SDE for 72 h. (D) Mast cells were isolated from the peritoneal cavity of rats. The rat peritoneal mast cells (RPMCs) were pretreated with 1–100 $\mu\text{g mL}^{-1}$ of SDE for 10 min, and then 5 $\mu\text{g mL}^{-1}$ of compound 48/80 was added to the cells within 10 min. The degranulation of mast cells was observed by microscopy. Each value is presented as the mean $\pm$ standard deviation. Bars represent a significant difference from the control at ## P < 0.01 or the only stimulation at * P < 0.05 and ** P < 0.01. Data were analyzed using analysis of variance followed by F-protected Fisher's least significant difference test.

treatment of SDE did not increase the expansion of CD4⁺Foxp3⁺ T cells (data not shown).

As the third anti-allergic mechanism, the SDE prevented the degranulation of mast cells. Compound 48/80 increases the permeability of the cellular membrane, and it is able to induce the release of histamine from mast cells by perturbing their membrane. The release of histamine by degranulation of mast cells can induce anaphylactic reactions, potentially even resulting in anaphylactic shock.³⁰ The mechanism underlying degranulation

of mast cells has been known to relate to activation of protein kinase C, which results in an increase in the uptake of extracellular Ca²⁺ into mast cells.³¹ Briefly, compound 48/80 phosphorylates cAMP-dependent proteins and then generates superoxide anions, one of the reactive oxygen species in mast cells. The superoxide anion activates inositol 1,4,5-triphosphate and increases the influx of Ca²⁺ into mast cells. To inhibit the release of histamine by degranulation, the role of free radical scavenger materials

were examined. Recently, it was reported that flavonoids, stilbenes, and curcuminoids, which are well-known antioxidants from medicinal plants, could act as degranulation inhibitors in antigen-stimulated rat basophilic leukemia (RBL-2H3) cells.^{32,33} In particular, among the flavonoids, quercetin, fisetin, luteolin, and ombuin significantly inhibited the degranulation of RBL-2H3 cells to an IC₅₀ of 4.6, 3.0, 3.0, and 5.5 $\mu\text{mol L}^{-1}$, respectively.^{34,35} Therefore, we analyzed the SDE by liquid chromatography mass spectrometry (LC-MS) to identify the active components of SDE for these effects. As a result, we confirmed that several types of chlorophyll were included in SDE such as chlorophyll C2, pyropheophorbide-a, and pheophorbide-b type (see Supporting Information Fig. S51). It was reported that chlorophyll C2 showed anti-allergic activity by suppressing degranulation of RBL-2H3 cell sensitized with anti-DNP-IgE. Furthermore, chlorophyll C2 inhibited IgE-mediated allergic responses in rhinitis mouse model³⁶ and decreased the rescue medication scores in clinical double-blind trial of seasonal allergic rhinitis.³⁷ Therefore, we suggest that SD including various types of chlorophyll shows potential as an anti-allergic agent for treatment of allergic disorders like food allergies.

CONCLUSIONS

We found that SDE ameliorated the symptoms of food allergy through the inhibition of allergen permeation and IL-4 production in the early allergic phase and also by suppression of mast cell degranulation in the late allergic phase. Although SD was ethnically used to treat inflammation and allergies, its efficacy had not yet been scientifically established. In the present study, we verified the anti-allergic effect of SDs on food allergies and provided scientific evidence between SD and allergy. We anticipate that SDs may be useful in the treatment/prevention of allergic disorders, including food allergies, as a therapeutic agent.

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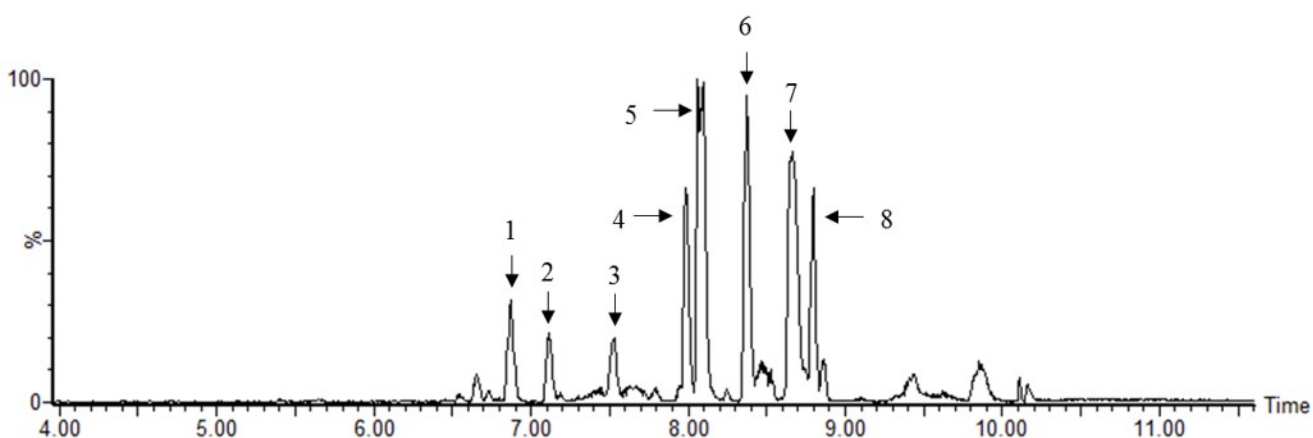
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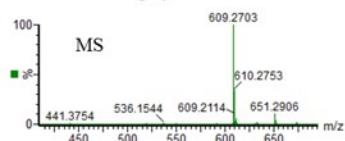
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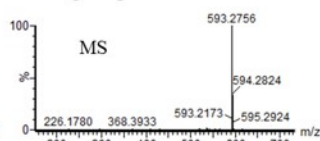
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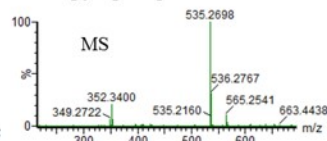
1. Chlorophyll C2



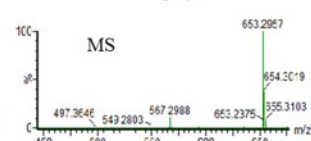
2. pheophorbide a



3. pyropheophorbide a



4. chlorophyll C3

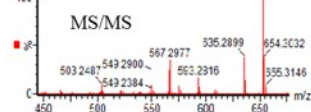
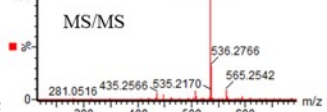
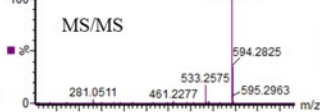
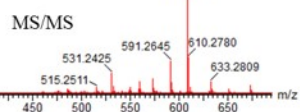


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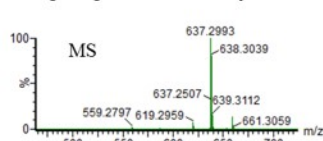
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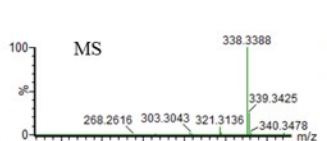
5. pheophorbide b methyl ester



6. pheophorbide b type



7. 13Z-docosenamide



8. chlorophyll derivate

