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Quillaja Saponin Can Modulate Ovalbumin-Induced IgE Allergic Responses through Regulation of Th1/Th2 Balance in a Murine Model

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Quillaja saponin is the extract from the balk of a South American tree, and it is considered to modulate immunological responses. We hypothesized that *Quillaja* saponin may change allergy-associated cytokine profile and antigen-specific immune responses. The purpose of this study is to investigate whether *Quillaja* saponin can suppress ovalbumin (OVA)-induced IgE-mediated allergic responses through promoting a dominant Th1 immune response. The spleen cells from BALB/c mice, which were primed by OVA, were used for an in vitro challenge test. The level of total and OVA-specific IgE, IL-4, IFN-γ, and IL-12 was determined by enzyme-linked immunosorbent assay (ELISA). BALB/c mice were orally administered with saponin for 35 days. The mice were immunized intraperitoneally with OVA on days 14 and 21. After intraperitoneal challenge with OVA on day 35, anaphylactic symptoms were monitored. Total and specific IgE and IgG, specific IgG1 and IgG2a, and histamine levels in serum were analyzed by ELISA. The increase of IL-12 and IFN-γ levels was observed in the presence of *Quillaja* saponin, while the IL-4 level was decreased. Furthermore, *Quillaja* saponin suppressed total and OVA-specific IgE secretion in spleen cells. Balb/c mice that were orally administered *Quillaja* saponin exhibited lower total and OVA-specific IgE and OVA-specific IgG secretions, whereas total IgG levels remained unchanged. Suppression of OVA-specific IgG1 and an increase of OVA-IgG2a were observed in mice fed saponin. *Quillaja* saponin also decreased serum histamine levels and diminished anaphylactic symptoms. The present study indicates that *Quillaja* saponin can suppress allergen-specific IgE-mediated reactivity in a murine model of food allergy, which results from shifting from a Th2-dominated to a Th1-dominated immune response.

KEYWORDS: Cytokines; IgE; IL-4; IL-12; INF-γ; *Quillaja* saponin; Th1/Th2; ovalbumin; food allergy; BALB/c mice

INTRODUCTION

Food allergy is a common health problem in developed countries, and it is thought to affect up to 1.5% of adults and 6% of children under 3 years of age (*1*). Most food allergic reactions are mediated by allergen-specific IgE antibodies. Thus, development of a method for inhibiting allergen-specific IgE production is a useful approach for preventing food-allergic diseases. IgE synthesis is considered to be due to the development and activation of Th2 cells and B cells. This specific Th2 cell produces predominantly IL-4 and IL-5. IL-4 plays a crucial role in inducing class switching of the IgE isotype and its production. IL-5 enhances IL-4-dependent IgE production (*2*, *3*). In contrast, the Th1 cells mainly secret cytokines such as IL-2 and IFN-γ, which inhibit IgE and IgG1 secretion and enhance IgG2a secretion (*4*−*8*). Thus, shifting the balance from Th2 to Th1 dominance should be a rational strategy for preventing IgE-mediated allergic diseases.

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Saponins are naturally occurring amphiphilic triterpene glycosides found in various plants. They consist of a lipophilic aglycone backbone with hydrophilic sugar side chains (*9*). Saponins have been reported to have a wide variety of biological properties, including hypocholesterolemic, immunostimulatory, antitumorogenic, and antimutagenic activities, depending on the structure (*10*, *11*). *Quillaja* saponin is the extract from the balk of a South American tree (*Quillaja saponaria molina*) and has two normonoterpene ester moieties, linked linearly to their fucosyl residue, which possess powerful adjuvant activities (*12*). Studies so far have demonstrated the immunomodulating properties of *Quillaja* saponin. Several reported that the immunostimulating complexes containing mainly *Quillaja* saponin (QH-A ISCOMs) activate T cells to produce high levels of IL-2 and IFN-γ in mice spleen cells (*13*−*16*). In addition, the QH-A ISCOMs have also been reported to induce the production of IL-4 and IL-5 as well as strong humoral responses, including high levels of IgG1 and IgG2a (*17*, *18*). These studies suggest that *Quillaja* saponin upregulates both Th1 and Th2 immune responses. Conversely, other studies showed that QH-A ISCOMs

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induce the prominent production of IFN-γ higher than that of IL-4 and IL-5 with a strong induction of IgG2a antibodies, suggesting induction of the pronounced Th1 profile (*19*, *20*). These findings suggest that *Quillaja* saponin induces primarily Th1 or balanced Th1/Th2 responses, depending on the antigen used. However, there is little information about the immunomodulatiory properties of *Quillaja* saponin against Th1/Th2 balance as an immunotherapy for food allergies.

In the present study, we examined the effect of *Quillaja* saponin on Th1/Th2 cytokine and IgE production by cultured spleen cells from ovalbumin (OVA)-sensitized BALB/c mine. We further studied the effect of orally administered *Quillaja* saponin on the OVA-induced allergic response, including immunoglobulin production, histamine release, and anaphylaxis-like symptoms.

MATERIALS AND METHODS

Mice, Saponin, and Antigen. Female BALB mice (6−8 weeks old) were purchased from Charles River (Mississauga, ON) and acclimatized for at least 1 week before the start of the study. Animal care and use were performed in accordance with the guidelines of Canadian Council on Animal Care. *Quillaja* saponin was obtained from Superfos Biosector (Vedbaek, Denmark). The antigen of OVA, 3-4,5-dimethylthiazol-2-yl-2,5 diphenyltetrazolium bromide (MTT), was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Cultures for OVA-Induced Cytokine and IgE Production in Vitro. The effect of saponin on OVA-induced cytokines and IgE production was studied using a culture system with OVA-primed mouse spleen cells. Briefly, BALB/c mice ($n = 5$) were sensitized with a subcutaneous injection of OVA (50 μg) and Freund's complete adjuvant in 50 μL of sterile saline. Spleen cells were collected from the spleen of the mice 7 days after OVA injection. The cells were washed and suspended in RPMI 1640 medium (Gibco BRL, Burlington, ON) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. The cells (1×10^6 cells/mL) were restimulated with OVA (25 μg) in the presence of saponin or saline in a 48 well culture plate (Corning, Cambridge, NY) and incubated at 37 °C in a CO_2 incubator for 3−10 days. Culture supernatants were harvested on day 3 or 10 for the determination of cytokine or IgE antibody levels, respectively. The viability of cells was evaluated using the MTT assay. Briefly, the adherent cells were added to 100 μL/well of MTT [5 mg/mL in phosphate-buffered saline (PBS)] (Sigma) and then incubated at 37 °C for 4 h before lysis in 10% sodium dodecyl sulfate in 0.01 M HCl. Absorbance at 570 nm was determined on each lysate, and cell viability was calculated by comparing results to control cells considered as 100% viable.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokines. The determination of IFN-γ, IL-4, and IL-12 in harvested culture supernatants was carried out by sandwich ELISA. For the measurement of IFN-γ and IL-4 levels, 96 well ELISA microtiter plates (Corning) were coated with 100 μL/well of rat anti-mouse IFN-γ or IL-4 monoclonal antibodies (0.2 μg/mL) (BD Bioscience, San Diego, CA) in 100 mM sodium phosphate buffer (pH 9.0) and incubated at 4 °C overnight. The plate was then washed three times with PBS containing 0.05% Tween-20 (PBST) and blocked with 200 μL/well of 2% bovine serum albumin (BSA) in PBS at 37 °C for 1 h. The plate was washed with PBST three times, and the culture supernatant samples (100 μL/well) were added into each well and incubated for 2 h at 37 °C. After the samples were washed four times, 100 μL/well of biotinylated rat anti-mouse IFN-γ or IL-4 monoclonal antibody (0.25 μg/mL) (BD Bioscience) in PBS containing 1% BSA was added and incubated for 1 h at 37 °C. The plate was further washed four times with PBST and incubated with avidin-horseradish peroxidase conjugate (100 μL/well, diluted 1:2000 in PBS containing 1% BSA) (BD Bioscience) for 1 h at 37 °C. The plate was washed six times and developed with 100 μL of 3,3′,5,5′-tetramethyl-benzidine (TMB) (Sigma) for 15 min at 37 °C. The reaction was terminated with 50 μL of 0.5 M H_2SO_4, and the absorbance at 450 nm was read by a microplate reader (Bio-Rad Laboratories, Hercules, CA). IL-12 (p70) was measured with ELISA kits (OptEIA set, BD Biosciences) in accordance with the manufacturer's instructions.

Oral Administration of *Quillaja* Saponin and OVA Sensitization in Mice. To investigate the effects of *Quillaja* saponin on the antigen-specific immune response and allergic reaction in vivo, animal tests using a murine food allergy model were performed. Briefly, BALB/c mice ($n = 5$ per group) were orally administrered *Quillaja* saponin (0.1 mg/head/time) in 0.1 mL of PBS by gavage three times a week throughout the experimental period for 35 days. The mice were immunized intraperitoneally with OVA (25 μg) in 100 μL of aluminum hydroxide gel adjuvant (alum) (2%, Alhydrogel, Westbury, CA) on days 14 and 21. An active systemic anaphylaxis challenge was elicited by intraperitoneal injection with OVA (2 mg) in 200 μL of PBS or PBS alone on day 35.

Assessment of Hypersensitivity Reactions. Anaphylactic symptoms were evaluated 40 min after challenge dose by using a scoring system as described by Li et al. (*21*) and were scored as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; and 5, death.

Measurement of Serum Histamine Levels. Forty minutes after intraperitoneal injection with OVA, mice were bled and the collected sera were frozen at −30 °C. Serum histamine levels were determined using an ELISA kit (Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany) in accordance with the manufacturer's manual.

ELISA for Immunoglobulins. Determination of total and OVA-specific IgG and IgE levels was performed by sandwich ELISA. For the measurement of total IgG and IgE, 96 well ELISA plates were coated with 100 μL/well of rat anti-mouse IgG (1 μg/mL) (Calbiochem, San Diego, CA) or IgE (Caltag, Burlingame, CA) monoclonal antibody (1 μg/mL), respectively, and incubated at 4 °C overnight. The plate was then washed with PBST three times and blocked by incubation with 200 μL/well of 2% BSA for 1 h at 37 °C. After the samples were washed with PBST three times, 100 μL of each culture supernatant sample (diluted 1:5000 for IgG and 1:100 dilution for IgE in PBST containing 1% BSA) was added into each well and incubated for 2 h at 37 °C. The plates were further washed four times with PBST, and then, 100 μL/well of alkaline phosphatase conjugated rabbit anti-mouse IgG (Sigma) (diluted 1:2000 in PBST containing 1% BSA) for measurement of the IgG levels was added. For measurement of the IgE level, 100 μL/well of monoclonal anti-mouse IgE conjugate to biotin (Caltag) (0.4 μg/mL in PBST containing 1% BSA) was added. All plates were incubated for 2 h at 37 °C. For the IgE assay, the plate was further incubated with 100 μL/well of Extra-Adivin alkaline phosphatese (Sigma) (diluted 1:3000 in PBST containing 1% BSA) for 1 h at 37 °C. The plate was washed six times and developed with 100 μL/well of *p*-nitrophenol phosphate (Sigma) in 0.1 M diethanolamine buffer (pH 9.8) for 1 h at 37 °C. The reaction was terminated with 50 μL/well of 3 N NaOH, and the absorbance at 405 nm was measured. For specific IgG and IgE measurements, 2 μg/well of OVA was coated instead of anti-mouse IgG and IgE anotibodies and the same procedures were followed.

For OVA-specific IgG1 and IgG2a determination, indirect ELISA was performed. OVA antigens were coated (5 μg/well), and subsequent steps were the same as above. Samples were diluted at 1:500 dilutions for IgG1 and 1:100 dilutions for IgG2a. Rat anti-mouse IgG2a and IgG1 (final concentration of 1 μg/mL) (100 μL) (BD Bioscience) were added to check the bound antibodies, and peroxidase-conjugated mouse anti-rat Ig (1:2000) (BD Bioscience) was added to detect the bound antibodies, and the reaction was developed with a TMB substrate-developing solution (Sigma) and stopped with 1 N H_2SO_4. The absorbance was measured at 450 nm.

Statistical Analysis. The statistical significance of the data was determined by Student's *t*-test. A *p* value of less than 0.05 was taken as significant.

RESULTS

Effect of *Quillaja* Saponin on OVA-Induced IgE and Cytokin Production in Vitro. To study whether IgE production

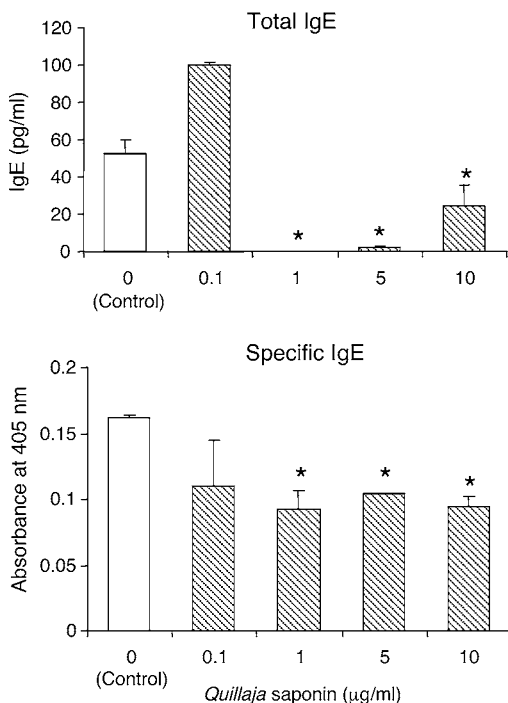

Figure 1. Total and specific IgE antibody levels in the culture supernatants of the splenocytes obtained from the OVA-immunized mice in the presence of *Quillaja* saponin. The asterisk indicates a significant difference from the control value (*$p < 0.05$). Bars represent the mean values (±SD) of triplicates.

is suppressed by *Quillaja* saponin in an in vitro culture system, spleen cells from OVA-sensitized BALB/c mice were cultured with OVA in the presence of *Quillaja* saponin or PBS, and the levels of total and OVA-specific IgE in culture supernatants were determined. As shown in **Figure 1**, total IgE production was significantly suppressed ($p < 0.05$) in the presence of 1, 5, and 10 μg/mL of *Quillaja* saponin, and OVA-specific IgE production was also inhibited by *Quillaja* saponin ($p < 0.05$). To determine the regulatory effect of *Quillaja* saponin on Th1/Th2 cell responses, IL-12, IFN-γ (Th1-associated cytokines), and IL-4 (Th2-associated cytokine) production in OVA-stimulated spleen cells was determined (**Figure 2**). The addition of *Quillaja* saponin to the cultures induced stimulation of IL-12 and IFN-γ production in a dose-dependent manner, while it inhibited IL-4 secretion. In addition, cell viability during the experimental incubation period was evaluated using the MTT assay and decreases of cell viability were not observed in the spleen cells in the presence of *Quillaja* saponin (data not shown).

Effect of Orally Administered *Quillaja* Saponin on OVA-Induced Allergic Reaction. We next examined whether the development of an antigen-specific immune response could be modulated by orally administered *Quillaja* saponin. Mice were orally administered 0.1 mg of *Quillaja* saponin or PBS (control) three times a week during the experimental period and immunized twice interperitoneally with OVA at an interval of 1 week. The group orally administered *Quillaja* saponin inhibited 51.5% of the total IgE antibody production in serum as compared with the control group, and a significant decrease ($p < 0.05$) in OVA-specific IgE production was also observed (**Figure 3**). In contrast, the OVA-specific IgG antibodies level in the saponin group was statistically lower ($p < 0.05$) than that in the control group, whereas a significant difference in total IgG levels was not observed ($p > 0.05$) (**Figure 4**). The specific IgG1 level in the saponin group (absorbance, 0.682) was lower than that that in the control group (absorbance, 2.06),

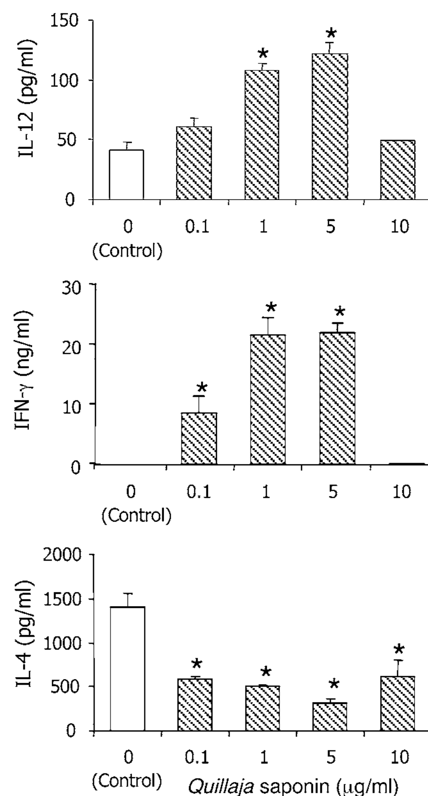

Figure 2. Cytokine measurement in the culture supernatants of the splenocytes obtained from the OVA-immunized mice in the presence of *Quillaja* saponin. The asterisk indicates a significant difference from the control value (*$p < 0.05$). Bars represent the mean values (±SD) of triplicates.

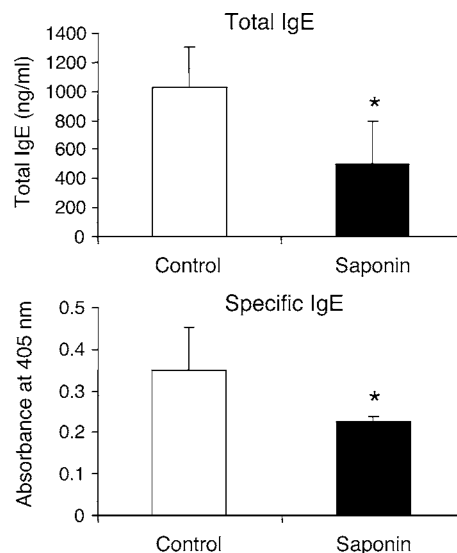

Figure 3. Effects of dietary *Quillaja* saponin on total IgE and OVA-specific IgE levels in immunized BALB/c mice. Each column represents the mean ± SE for individual mice ($n = 5$). The asterisk indicates significant differences at $p < 0.05$ between two groups.

although the specific IgG2a level in the saponin group (absorbance, 0.123) was higher than that in the control group (absorbance, 0.077) (**Figure 5**).

The effect of orally administered *Quillaja* saponin on food-allergic reactions induced by allergen exposure was assessed. Mice (five per group) immunized with OVA during oral administration of *Quillaja* saponin were challenged intraperitoneally with 2 mg of OVA. In the control group, systemic

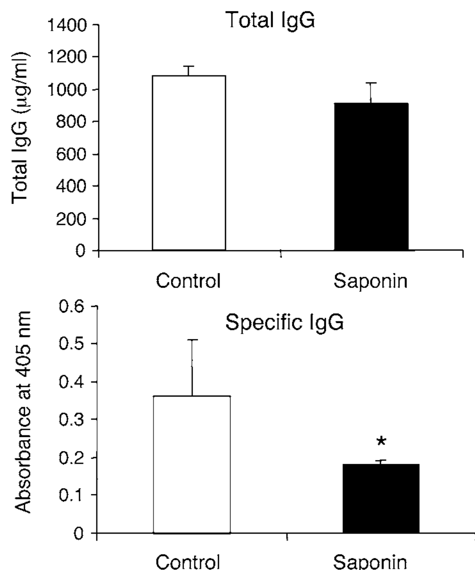

Figure 4. Total IgG and OVA-specific IgG levels in serum in control and *Quillaja* saponin-fed BALB/c mice. Each column represents the mean ± SE for individual mice. The asterisk indicates significant differences at $p < 0.05$ between two groups.

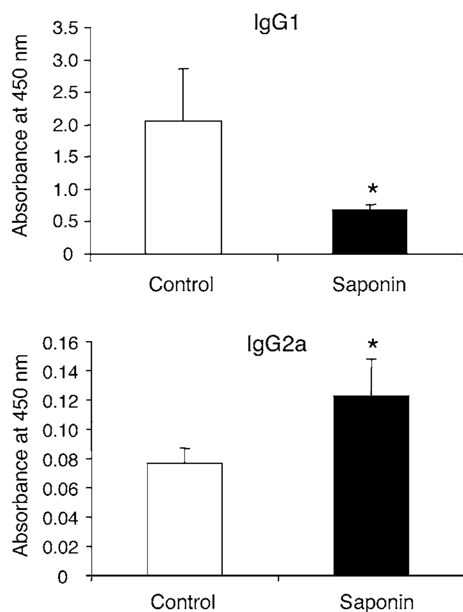

Figure 5. Determination of specific IgG1 and IgG2 levels in serum in control and *Quillaja* saponin-fed BALB/c mice. Each column represents the mean ± SE for individual mice. The asterisk indicates significant differences at $p < 0.05$ between two groups.

anaphylactic symptoms developed within 40 min (average score, 3.2). In contrast, the symptom average score reduced to 1.2 in mice of the saponin group (**Figure 6A**).

It is known that IgE-mediated degranulation of mast cells plays a major role in the immediate allergen-induced anaphylactic reactions and histamine is a key anaphylactic mediator released by mast cells (*22*). The level of serum histamine was measured after oral challenge. As shown in **Figure 6B**, the histamine level in the control group was 900.7 ng/mL; however, when the mice were orally administrated *Quillja* saponin, the histamine level dropped to 176.9 ng/mL.

DISCUSSION

Helper T cells are classified into two types (Th1 and Th2), based on production patterns of particular cytokines. Two types

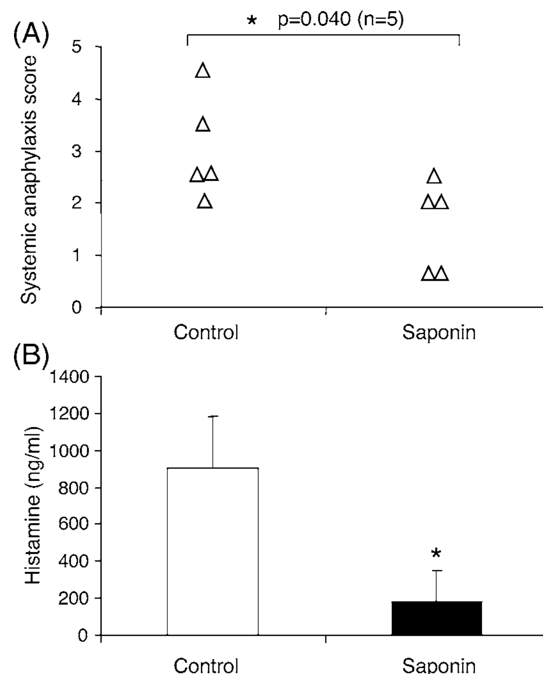

Figure 6. Anaphylactic responses after oral challenge in mice presensitized in the presence of *Quillaja* saponin. Groups of mice that had received orally administered *Quillaja* saponin or PBS during sensitization to OVA (five per group) were challenged intraperitoncally. (**A**) Symptom scores of systemic anaphylaxis. The anaphylaxis-like symptoms were scored within 40 min after oral challenge, on a scale from 0 (no symptoms) to 5 (death), as described in the Materials and Methods. Open triangles indicate individual mice. (**B**) Serum histamine levels. Forty minutes after oral challenge, mice were bled and aliquots of serum were collected. Data are reported as means ± SD; $^*P < 0.05$.

of T cells maintain well-balanced relations in the modulation of cytokine secretion to keep homeostasis in the host, and disruption of this balance induces various immunological diseases. Allergic disease is characterized as an excessive Th2 type immune response. It is generally accepted that IL-4 regulates the differentiation of native CD4 + T cells into helper Th2 cells and the immunoglobulin class switching to the IgG1 and IgE isotypes. Excessive IL-4 production by Th2 cells has been associated with an elevation of IgE levels and allergic reaction. Thus, modulation of the Th1/Th2-balanced immune response is one of the most strategic immunotherapies for allergic diseases (*23*, *24*). Previous studies have shown that *Quillaja* saponin can enhance the cellular immunity of the host through promoting the Th1 type immune response (*19*, *20*). These findings led us to hypothesize that *Quillja* saponin may inhibit IgE production through shifting from the Th2 type immune response toward the Th1 type.

In the present study, we first showed that the amounts of Th1 type cytokine, IFN-γ, produced by spleen cells exposed to OVA and *Quillaja* saponin were significantly higher than those exposed to OVA alone, while the amounts of Th2 type cytokine, IL-4, produced by spleen cells with OVA and *Quillaja* saponin, were lower than those with OVA alone. Also, augmentation of IL-12 production was observed in the cultures in addition to *Quillaja* saponin. IL-12, produced by antigen-presenting cells during the initial stages of an immune response, plays a pivotal role in the induction of IFN-γ by NK and $\gamma\ \delta$ T cells and in driving the differentiation of Th1 cells (*25*, *26*). These findings suggest that *Quillaja* saponin could stimulate IL-12 production from antigen-presenting cells and then shift toward the Th1 immune response. Furthermore, in our culture system of murine

spleen cells primed by antigen immunization, diminished production of antigen-specific IgE was observed in response to treatment with *Quillaja* saponin. Thus, the findings obtained from this in vitro study suggest that *Quillaja* saponin is effective in inhibiting the IgE antibody response by skewing the pattern of cytokine production toward Th1 dominance.

We next tried to evaluate the effect of orally administered *Quillaja* saponin on suppressing allergic reactions in an in vivo food allergy model. We showed that *Quillaja* saponin administration to mice suppressed systemic anaphylaxis induced by antigen stimulation and that the increase in serum histamine of the *Quillaj* saponin group was lower as compared with that of the control group. Also, secretion of Th2-driven IgE and IgG1 was markedly inhibited by oral administration of *Quillaja* saponin, whereas secretion of Th1-driven IgG2a was increased. Although we did determine the cytokine levels in *Quillaja* saponin-administered mice, it is suggested that *Quillaja* saponin administration could shift the pattern of cytokine production in splenocytes toward Th1 dominance according to the data obtained from the in vitro cell culture study. These findings imply that the reduction of systemic anaphylaxis is considered to result from the inhibition of OVA-specific IgE antibody response through shifting of the balance from Th2 to Th1 dominance.

Several groups have also demonstrated that saponin has antiallergic activity. Park et al. showed that intravenous administration of saikosaponin inhibited the passive cutaneous anaphylaxis reaction in OVA-immunized rats, and it inhibited histamine release in rat mast cells (*27*). Furthermore, Tachibana et al. reported that soybean saponins inhibited histamine release in human basophilic cells through the suppression of the cell surface expression of a high affinity IgE receptor (*28*). These reports suggest that saponins inhibit histamine release through inhibiting activation of mast cells. In this study, we did not study the effect of *Quillaja* saponin on mouse mast cell inactivation, but it is obvious that it suppressed anaphylaxis reaction by means of saponin feeding, which might be associated with mast cell function. In our culture system of murine splenocytes, the diminished production of IgE and IL-4 was observed with respect to treatment of *Quillaja* saponin. Thus, we assume that the inhibitory effect of histamine release by oral administration of *Quillaja* saponin was likely due to suppression of the Th2 immune response; however, *Quillaja* saponin might directly affect the IgE receptor on the mast cells. The mechanism underlying the antiallergic activity of *Quillaja* saponin, including mast cell funcitons, is now being explored at cellular and molecular levels in our laboratory.

Recent reports have shown that dietary ingredients exhibit the capacity to reduce antiallergen IgE production through the modulation of the Th1/Th2 balance. Shida et al. demonstrated that dietary *Lactobacillus casei* strain Shirota to mice downregulates the serum IgE production against OVA through suppression of the Th2 type immune response (*29*). It has been reported that the feeding of chitin, a polymer of *N*-acetyl-D-glucosamine, downregulates serum IgE levels in mice through the induction of a Th1 response (*30*). Chondroitin sulfate, a homopolymeric glycosaminoglycan, inhibited OVA-specific IgE production through induction of cytokine secretion by Th1 cells in the murine splenocytes culture systems (*31*). Our present study showed that *Quillaja* saponin, amphiphilic triterpene glycosides, can modulate the pattern of the Th1/Th2 immune response in OVA-sensitized mice. To date, 30 different kinds of saponin have been identified with different structures and further studies using different structural saponin would be necessary to clarify the relation of structure and antiallergic functions of saponin.

In conclusion, we demonstrated that *Quillaja* saponin can suppress the allergen-specific IgE-mediated allergic reaction in a murine model of food allergy, which resulted from shifting from a Th2- to a Th1-dominated immune response. The present study suggests a possible use of *Quillaja* saponin in preventing IgE-mediated allergy. To use it clinically for the prevention of allergic disease, the optimal dosage, efficacy, and adverse effects in humans should be determined.

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