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Suppression of Allergic Reactions in Ovalbumin-Sensitized Mice by Yam Storage Proteins Dioscorins

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ABSTRACT: To study the biomedical functions of dioscorins isolated from various species of *Dioscorea*, we investigated their antiallergic potential using an OVA-induced allergy mouse model. All the dioscorins suppressed allergic reactions by decreasing the serum IgE and histamine levels. The serum IFN-γ and IgG2a levels increased in all the dioscorin-treated mice. The spleen cells from the dioscorin-treated mice also exhibited an up-regulation of IFN-γ secretion in response to ConA stimulation. Although dioscorins did not affect the IgG1 levels, the IL-5 levels decreased to basal levels in mice treated with dioscorins of *D. alata* or *D. japonica* and in most of the lymphoid cells of the dioscorin-treated mice in response to ConA stimulation. The decrease of IgE and histamine levels was concomitant with an increase in IFN-γ and IgG2a levels and with a decrease in IL-5 levels, suggesting that dioscorins suppressed the OVA-induced allergic reactions, possibly through modulating an imbalanced Th1/Th2 immune response.

KEYWORDS: *dioscorin, yam, storage protein, IgE, histamine, allergy, Th1, herbal medicine*

INTRODUCTION

IgE-mediated hypersensitivity (type I allergy) is the most common form of allergy in industrialized countries.[1] The allergic symptoms caused by type I hypersensitivity reactions include atopic disease, allergic rhinitis, and bronchial asthma. Type I allergic responses involving IgE-dependent mast-cell degranulation and eosinophil accumulation in inflammation sites are considered to be due to the development and activation of Th2 cells.[2−5] Differentiation of allergen-specific Th2 cells leads to the production of IL-4 and IL-13, which induce immunoglobulin class switching to IgE production in B lymphocytes, and also leads to the production of IL-5, which regulates differentiation and activation of eosinophils, and IL-9, which enhances mast-cell differentiation.[3,5,6] Binding of an allergen to the mast cell and basophil cell-bound IgE leads to degranulation of vasoactive amines (mainly histamine), lipid mediators (prostaglandins and leukotrienes), chemokines, and cytokines (IL-4, IL-5, and IL-13), which are responsible for the immediate phase of allergic reactions.[2−4]

During allergy alleviation, Th1 cell subsets produce IL-2 and IFN-γ. IFN-γ amplifies Th1 cell development and inhibits the proliferation and activation of Th2 cells. IFN-γ has been shown to suppress the production of IgG1 and to increase the production of IgG2.[2,7,8] These two cytokines also suppress the production of IL-4 and IL-5 by resting Th2 cells, inhibit the production of eosinophils and the secretion of IgE and histamine, and increase the production of IgG2a to further suppress allergic reactions.[9] Therefore, allergen-induced Th1/Th2 cell imbalance has been suggested to be responsible for inflammation in allergic responses.[10]

Yam (*Dioscorea spp.*) tubers are highly nutritional and widely consumed in Africa and Asia. Yam tubers have functional components such as mucin, dioscin, diosgenin, allantoin, choline, polyphenol oxidases, and proteins.[11] Dried slices of yam tubers have been used in Chinese herbal medicine since ancient times to strengthen the functions of the spleen, kidney, liver, and stomach, to reduce phlegm, and to heal fatigue, chronic diarrhea, and diabetes.[12] Yam tubers contain approximately 1 to 3% of proteins on a dry-weight basis.[13] Dioscorins, the major storage proteins in yam tubers, account for approximately 85% of the total protein content of the tubers. Dioscorins of various yam species contain two isoforms, Class A and Class B.[14,15] Class A dioscorin is a monomer (32 kDa), whereas Class B dioscorin is a dimer (66 kDa).

Dioscorins exhibit both biochemical and immunomodulatory activities. Dioscorins have been shown to possess carbonic anhydrase, as well as free radical scavenging activities, angiotensin-converting enzyme inhibiting activities, antihypertensive activities, and trypsin-inhibiting activities.[16−20] Dioscorins can activate the Toll-like receptor-4 signaling pathway and stimulate the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in RAW264.7 macrophages, murine bone marrow cells, and human monocytes ex vivo.[21−23] Moreover, dioscorins from *D. alata* induced the expression of the nitric oxide synthase gene, which resulted in the accumulation of nitric oxide and the increase in the oxidative burst in RAW264.7 macrophages.[22] However, dioscorins from different species may exhibit distinct immunomodulatory activities in mice. Lin et al. (2009) showed that dioscorins from *D. alata* had a higher ability than those from *D. japonica* for stimulating the phagocytic activity of lymphoid cells; however, dioscorins from *D. japonica* were more effective than those from *D. alata* in enhancing the proliferation of lymphoid cells.[23] Oral administration of dioscorins from *D. alata* to BALB/c mice stimulated the IFN-γ, IL-4, and IL-10 secretion

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of splenocytes and increased the subpopulation in NK cells and B cells, as well as the numbers of Peyer's patches.[24] Furthermore, dioscorins from *D. japonica* and *D. alata* were shown to protect the tight junction protein (zonula occludens, E-cadherin, and desmoplakin) expression in human airway epithelium cells from dust mite damage, suggesting that dioscorins are a potential protector against airway damage caused by mites.[25]

Although dioscorins have exhibited immunomodulatory activities in mice, their ability to suppress allergic reactions has not been studied yet. In the present study, we used the BALB/c mouse strain and the well-characterized ovalbumin (OVA) sensitization model to evaluate the suppressing effect of dioscorins against allergic reaction in the OVA allergy mouse model. We investigated their in vivo effect on IgE and histamine production, as well as Th1/Th2 cytokine and IgG2a/IgG1 antibody production.

MATERIALS AND METHODS

Plant Materials. The fresh yam tubers of Japanese yam (*Dioscorea japonica* Thunb.), *D. alata* var. Tainong No.1, and *D. pseudojaponica* var. Keelung were routinely purchased from a farmer association (Mingchien Shiang, Nantou County, Taiwan). All yam tubers were stored in a cooler controlled at 16 °C before use.

Experimental Animals. Seven-week-old, inbred, and certified male BALB/c mice (approximately 20−25 g) were purchased from BioLASCO (Taipei, Taiwan). Mice were allowed to acclimatize for 7 days upon arrival and allocated randomly into treatment groups. Mice were maintained on a 12/12 h light/dark cycle at 22 ± 2 °C and provided with food (PMI Nutrition International, Missouri, U.S.A.) and water at the Institute of Biotechnology, National Dong Hwa University, Taiwan. All mice were housed and cared according to the "Guide for the Care and Use of Laboratory Animals" of National Dong Hwa University.

Extraction and Purification of Dioscorin Proteins. The dioscorins were extracted and purified according to the procedure of Lin et al. (2009).[23] Briefly, approximately 500 g of yam tubers was peeled, sliced, and blended with 4 vols (w/v) of 50 mM Tris-HCl (pH 8.3). After centrifugation at 12 000*g* for 30 min, total proteins of the crude extract were differentially precipitated with 45−75% ammonium sulfate and dialyzed overnight with Cellu·Sep T4 (MW 12 000−14 000, Regenerated Cellulose Tubular Membrane, Seguin, Texas, U.S.A.) against 50 mM Tris-HCl buffer (pH 8.3). Approximately 500 mL of the dialyzed protein sample was mixed with 5 g ddH_2O-hydrated DEAE Sephadex A-25 ion exchange (Amersham Pharmacia Biotech, Uppsala, Sweden). The precipitate was collected after centrifugation at 10 000*g* for 10 min and redissolved in 50 mL of 50 mM Tris-HCl (pH 8.3) buffer containing 150 mM NaCl. The protein solution was subsequently loaded onto a Sephadex G-75 column (C16/70, Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted with 100 mM Tris-HCl (pH 7.9) containing 100 mM NaCl. The eluate was dialyzed overnight with ddH_2O at 4 °C and then lyophilized. For the experiment, the lyophilized dioscorins were dissolved in phosphate-buffered-saline (PBS, pH 7.4), centrifuged at 12 000*g* for 20 min, and filtrated with 0.45 μm filter membrane (Millipore, Massachusetts, U.S.A.). Concentrations of the dioscorins were determined by Protein Assay Dye Reagent Concentrate (Bio-Rad, California, U.S.A.) and were further confirmed by SDS-PAGE loaded with standard concentrations of bovine serum albumin (Sigma, Missouri, U.S.A.). The purity of the dioscorins was determined by scanning the SDS-PAGE with luminescent image analyzer (Fujifilm LAS-3000, Tokyo, Japan). Based upon measuring the monomers of the dioscorins, approximately 89− 97% purity was obtained. The dioscorins isolated from *D. alata*, *D. japonica*, and *D. pseudojaponica* were designated as Da-dioscorins, Dj-dioscorins, and Dp-dioscorins, respectively.

OVA Sensitization and Dioscorin Injection Procedures. Eight-week-old mice were sensitized with 20 and 50 μg ovalbumin (OVA, Sigma, Missouri, U.S.A.) by intraperitoneal (ip) injection on day 1 and 14, respectively, according to the vaccination scheme described by Lin et al (2006).[26] The OVA was mixed with 2 mg of aluminum hydroxide (Sigma, Missouri, U.S.A.) in a total volume of 0.2 mL of phosphate-buffered-saline (PBS). Negative-control mice were given an ip injection of 0.2 mL PBS and referred to as the unimmunized group. The OVA-sensitized mice were ip injected with 2 mg/kg of various dioscorins on day 7, 15, 18, 21, and 24. The OVA-sensitized mice received PBS instead of dioscorins and served as positive controls to maintain hypersensitivity. On day 27, the mice were sacrificed by cervical dislocation. The body weight of the mice was 24.2 ± 1.1 g for the PBS group, 25.0 ± 1.6 g for the OVA group, 25.2 ± 1.5 g for the Da-dioscorin group, 25.5 ± 1.3 g for the Dj-dioscorin group, and 25.3 ± 1.6 g for the Dp-dioscorin group. Mice were bled from the plexus retroorbitalis, and sera were stored at −20 °C until determination of indicated parameters.

Isolation of Bone Marrow, Spleen, and Thymus Cells from Mice. After sacrifice of the mice, the thighbone, spleen, and thymus were immediately removed, cleaned of fat and connective tissue, and placed in PBS. Tissues (thymus, spleen, and bone marrow) were then individually cut into small pieces and were forced through the nylon mesh (mesh size 80) to obtain a cell suspension. The immune cell suspension was washed with PBS buffer three times and resuspended with RPMI-1640 medium containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cell numbers isolated from the tissues were counted with a hemacytometer. The cells were used for analyzing cytokine production and cell proliferation.

Measurement of Serum Histamine Levels. The levels of serum histamine were determined by competitive direct enzyme-linked immunosorbent assay (ELISA) (Oxford Biomedical Research, Inc., Michigan, U.S.A.) following the manufacturer's protocol. Briefly, 96-well plates (SPL, Gyeonggi-Do, Korea) were coated with 100 μL of mouse histamine monoclonal antibody (10 μg/mL in 0.05 M carbonate−bicarbonate, pH 9.6). Fifty microliters of reference or diluted sample sera (1:1000 dilution) were added to the wells. The wells were then added with horseradish peroxidase-labeled secondary antihistamine antibody and cultured for 45 min. After washing the wells with 300 μL of wash buffer three times, 100 μL of tetramethylbenzidine (TMB substrate) was added to the wells. After incubating the wells for 30 min, 100 μL of stop solution (2 M H_2SO_4) was added into each well to stop the reaction. Absorbance at 450 nm was recorded with an ELISA plate reader (SpectraMax 190, Nihon Molecular Devices, Tokyo, Japan), and the actual amount of sample antibodies in sera was determined by quantifying the amount according to the reference sera.

Determination of Total Serum IgE, IgG1, and IgG2a Levels. Blood obtained on Day 27 was centrifuged at 3000*g* for

15 min. The sera were collected and stored at −80 °C for future assays. The titers of the total IgE, IgG1, and IgG2a were determined by sandwich ELISAs according to the manufacturer's instruction (Bethyl Laboratories, Inc., Montgomery, TX). Briefly, 96-well plates were coated with 100 μL purified mouse anti-IgE, anti-IgG1, or anti-IgG2a antibodies (10 μg/mL in 0.05 M carbonate−bicarbonate, pH 9.6) for 1 h at room temperature, and the wells were washed with 200 μL wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) for three times. The wells were then blocked with 200 μL of blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 30 min and washed with 200 μL wash buffer three times. Reference or diluted sample sera (1:10 dilution for IgE, 1:100 dilution for IgG1 and IgG2a) was added to the wells, and the wells were cultured for 1 h. After washing the wells with wash buffer three times, the wells were added with 100 μL of diluted HRP detection antibody (1:50000 dilution for IgE, 1:100000 dilution for IgG1, 1:10000 dilution for IgG2a) and incubated for 1 h. After incubation, the wells were washed with wash buffer three times, and 100 μL of TMB substrates were added to the wells. After incubating the wells for 30 min, 100 μL of stop solution (2 M H_2SO_4) was added into each well to stop the reaction. Absorbance at 450 nm was recorded with an ELISA plate reader (SpectraMax 190, Nihon Molecular Devices, Tokyo, Japan), and the actual amount of sample antibodies in sera was determined by quantifying the amount according to the reference sera.

Quantification of IL-2, IL-5, and IFN-γ Levels in Sera or Tissues. For determining the serum IL-5 and IFN-γ levels, blood obtained on day 27 was centrifuged at 3000*g* for 15 min. The sera were collected and stored at −80 °C for future assay. For quantifying the IL-2, IL-5, and IFN-γ production in tissues, approximately 2×10^5 bone marrow, spleen, or thymus cells were seeded in a 96-well plate and treated with 100 μL RPMI-1640 medium containing 50 μg OVA or ConA for 48 h. After incubation, the supernatant was collected and stored at −80 °C for future assay. Quantification of cytokine levels were performed by using the sandwich ELISA kit purchased from Bender Medsystem (Vienna, Austria) for IL-2 and IL-5 and Perprotech (New Jersey, U.S.A.) for IFN-γ. Briefly, the plate was coated with 100 μL (100 ng) of anticytokine antibody (1:100 dilution). After incubation overnight at 4 °C, the wells were washed with 200 μL of wash buffer three times. Each well received 250 μL of blocking solution and was incubated for 2 h. After washing the wells three times, the wells were cultured with 100 μL of standard or sample (1:10 dilution) for 1 h at room temperature. After washing, 50 μL of biotin-conjugated secondary antibodies was added to the wells and incubated for 2 h. After washing the wells, 100 μL of streptavidin−HRP was added to the wells and incubated for 1 h. After washing the wells, 100 μL of TMB substrate was added to the wells and incubated for 30 min. The enzyme reaction was stopped by adding 100 μL of stop solution into each well. Absorbance at 450 nm was recorded with an ELISA plate reader (SpectraMax 190, Nihon Molecular Devices, Tokyo, Japan), and the actual amount of cytokines was determined by quantifying the amount according to the standard.

Proliferation Assay of Immune Cells in Response to OVA, ConA, or Various Dioscorins. The bone marrow, spleen, and thymus cells were collected from the OVA-sensitized mice followed by ip injection with Da-dioscorins, Dj-dioscorins, or Dp-dioscorins. Approximately 2×10^5 cells/well in 100 μL of RPMI-1640 medium were seeded into a 96-well plate. The cells were treated with 50 μg (in 10 μL) of OVA, Da-dioscorins, Dj-dioscorins, or Dp-dioscorins for 48 h. After incubation, the culture medium was removed and replaced with 100 μL of PBS buffer. Proliferation of immune cells in response to supplemental OVA and dioscorins was assessed in triplicates by CellTiter 96 AQuenous Non-Radioactive Cell Proliferation Assay according to the manufacturer's recommendation (Promega, Wisconsin, U.S.A.). The assay reagents, 333 μg/mL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS, Promega, Wisconsin, U.S.A.) and 25 μM phenazine methosulfate (PMS, Sigma, Missouri, U.S.A.) were added to each well of the 96-well plate, and the plate was incubated at 37 °C for 2 h. Absorbance at 490 nm was recorded with an ELISA plate reader (SpectraMax 190, Nihon Molecular Devices, Tokyo, Japan). The values were calculated as relative intensity of the absorbance as compared to the vehicle control with no added mitogen.

Statistical Analysis. Results of three separate experiments performed in triplicates for the in vitro studies, or five mice (n = 5) for each group of the in vivo studies were expressed as mean ± SD. Treatments were analyzed for significant differences using one-way ANOVA, unless otherwise stated. SigmaStat statistical software of Windows version 2.03 (SPSS, Inc., Chicago, IL) was used to analyze the results. Differences between the positive control (OVA-sensitized mice) and the other groups of dioscorin-treated mice were considered statistically significant if $P < 0.05$ (*).

RESULTS

Effect of Dioscorins on the Suppression of IgE and Histamine Production. To determine whether dioscorins could suppress an allergic reaction, we sensitized BALB/c mice with ovalbumin (OVA), followed by ip injection of dioscorins from various yam species, over a 27 day period (Figure 1). The

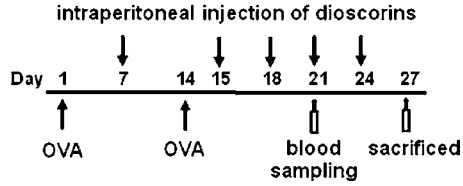

Figure 1. Experimental timeline of treatment. Groups of BALB/c mice (n = 5 per group) were sensitized two times by OVA followed by ip injection of various dioscorins on day 7, 15, 18, 21, and 24.

healthy mice that were ip injected with phosphate-buffered saline (PBS) throughout the experiment were referred to as the negative control. The OVA-sensitized mice that were ip injected with PBS served as a positive control to maintain hypersensitivity. We collected the sera from mice at 21 and 27 days after OVA immunization, followed by ip injection with dioscorins from various yam species, and determined the IgE and histamine levels in sera to evaluate the suppressive effect of dioscorins against the allergic reactions caused by OVA. Figure 2A shows that the total IgE levels in the OVA-sensitized mice (positive control) were 261 and 263 μg/mL on Day 21 and 27, respectively. This result was approximately 8-fold higher than those in the negative control mice (30−36 μg/mL), indicating that the OVA antigen induced an allergic reaction in the OVA-allergy mouse model. Multiple ip injections of various dioscorins into the OVA-sensitized mice for 21 or 27 days

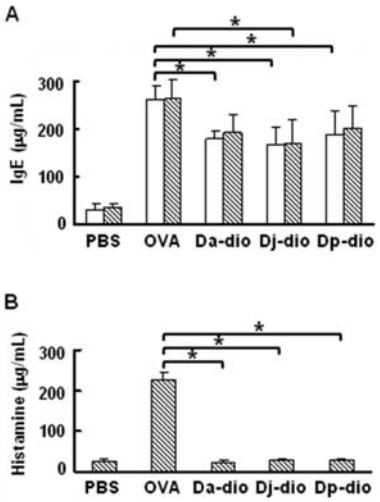

Figure 2. Effects of various dioscorins on the production of IgE and histamine in OVA-sensitized mice. OVA-sensitized BALB/c mice were ip injected with Da-dioscorins, Dj-dioscorins, or Dp-dioscorins. The sera at 21 (plain columns) or 27 days (shaded columns) after OVA sensitization were collected for measuring the IgE levels (A). The sera at 27 days after OVA sensitization were collected for determining the histamine levels (B). Three replicates for each measurement were performed. Results represent the mean ± SD of five mice (n = 5). Asterisk (*) represents a significant difference ($p < 0.05$) of the IgE or histamine levels between dioscorin-treated and OVA-sensitized mice.

significantly decreased the IgE levels in the sera, compared with the positive control mice.

We also monitored the histamine levels in the sera of OVA-sensitized mice that had been ip injected with various dioscorins. As shown in Figure 2B, the histamine levels in the positive control mice significantly increased to 226 μg/mL, 10-fold higher than those in the negative control mice (27 μg/mL), again indicating that the OVA antigen induced an allergic reaction in the mice. After ip injection with various dioscorins, the histamine levels significantly decreased to basal levels (22−29 μg/mL) in all the dioscorin-treated mice.

Effects of Dioscorins on the Th1 and Th2 Cell Responses. The development and activation of Th2 cells, which secrete IL-4 and IL-5 cytokines, are involved in allergic responses. These two cytokines may cause IgE-dependent mast-cell degranulation, eosinophil accumulation, and IgG1 secretion.[2,6] By contrast, during allergy alleviation, Th1 cells are activated to produce IFN-γ and IL-2.[2,5] These two cytokines inhibit the production of eosinophils and the secretion of IgE but increase IgG2a production to alleviate allergic reactions.[7−9] Therefore, the allergen-induced Th1/Th2 cell imbalance has been suggested to be responsible for inflammation in allergic responses.[10]

To elucidate whether dioscorins had a regulatory effect on the Th1 and Th2 cell responses in OVA-sensitized mice, we determined the levels of IFN-γ and IL-5 in the sera of OVA-sensitized mice after ip injection with various dioscorins. Figure 3A shows that the level of IFN-γ in the positive control mice (231 pg/mL) was comparable to that in the negative control mice (215 pg/mL). However, the level of IFN-γ significantly increased to 1078, 2214, or 3180 pg/mL after mice were ip injected with Da-dioscorins, Dj-dioscorins, or Dp-dioscorins, respectively. The levels were 5- to 14-fold higher than the IFN-γ level in the positive control mice. The IL-5 level increased to 48 pg/mL in the positive control mice, 3-fold higher than that

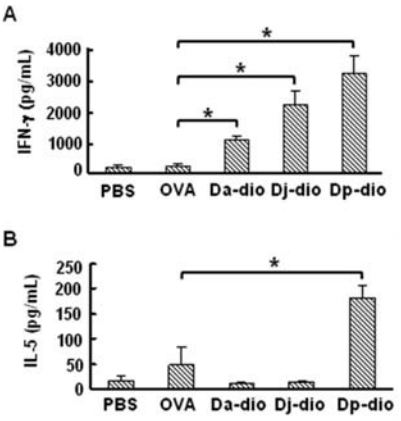

Figure 3. Effects of various dioscorins on the production of IFN-γ and IL-5 in OVA-sensitized mice. The sera at 27 days after OVA sensitization followed by dioscorin treatment were collected for measuring the IFN-γ (A) and IL-5 (B) levels. Three replicates for each measurement were performed. Results represent the mean ± SD of five mice (n = 5). Asterisk (*) represents a significant difference ($p < 0.05$) of the IFN-γ or IL-5 levels between dioscorin-treated and OVA-sensitized mice.

in the negative control mice (16 pg/mL) (Figure 3B). The IL-5 level dramatically decreased to basal levels (10−13 pg/mL) after the mice were ip injected with Da-dioscorins or Dj-dioscorins, whereas it significantly increased to 183 pg/mL in Dp-dioscorin-treated mice, nearly 4-fold higher than that in the positive control mice.

To further study the Th1 and Th2 cell responses in OVA-sensitized mice after ip injection with various dioscorins, we measured the IgG2a (secreted by Th1 cells) and IgG1 (secreted by Th2 cells) levels in the sera of dioscorin-treated mice. Figure 4A shows that the levels of IgG2a in dioscorin-treated mice were approximately 1.4- to 4-fold higher than those in the positive control mice. The levels of IgG1 in Da-dioscorin-treated or Dj-dioscorin-treated mice were comparable to those in the positive control mice (Figure 4B). Furthermore, the level of IgG1 in Dp-dioscorin-treated mice significantly

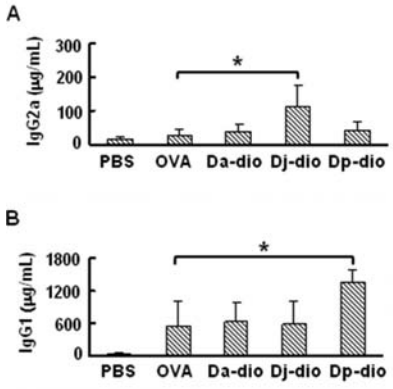

Figure 4. Effects of dioscorins on the production of IgG1 and IgG2a in OVA-sensitized mice. The sera at 27 days after OVA sensitization followed by dioscorin treatment were collected for measuring the IgG2a (A) and IgG1 (B) levels. Three replicates for each measurement were performed. Results represent the mean ± SD of five mice (n = 5). Asterisk (*) represents a significant difference ($p < 0.05$) of the IgG1 or IgG2a levels between dioscorin-treated and OVA-sensitized mice.

Table 1. Effect of Dioscorins on IFN-γ Production of Lymphoid Cells Stimulated with ConA[a]

		mice (pg/mL)				
treatment	lymphocytes	PBS	OVA	Da-dio	Dj-dio	Dp-dio
ConA	spleen	5587 ± 456*	1310 ± 347	3750 ± 803*	2276 ± 340	2945 ± 144
	bone marrow	337 ± 86	206 ± 14	193 ± 11*	209 ± 13	206 ± 12
	thymus	242 ± 31	294 ± 96	207 ± 1	310 ± 84	228 ± 34

[a]Lymphoid cells of the treated mice were stimulated with ConA, and the levels of IFN-γ were determined using ELISA assay. The difference between the results of indicated groups (PBS or dioscorin-treated mice) and the positive control (OVA-sensitized mice) was determined by the Student's *t* test, and a *P* value of less than 0.05 was considered to be statistically significant (*).

Table 2. Effect of Dioscorins on IL-2 Production of Lymphoid Cells Stimulated with ConA[a]

		mice (pg/mL)				
treatment	lymphocytes	PBS	OVA	Da-dio	Dj-dio	Dp-dio
ConA	spleen	467 ± 8*	382 ± 9	341 ± 1*	279 ± 4*	303 ± 13*
	bone marrow	833 ± 48	605 ± 75	538 ± 28	502 ± 6	523 ± 11
	thymus	332 ± 45*	466 ± 2	392 ± 13*	733 ± 6*	604 ± 2*

[a]Lymphoid cells of the treated mice were stimulated with ConA, and the levels of IL-2 were determined using ELISA assay. The difference between the results of indicated groups (PBS or dioscorin-treated mice) and the positive control (OVA-sensitized mice) was determined by the Student's *t* test, and a *P* value of less than 0.05 was considered to be statistically significant (*).

Table 3. Effect of Dioscorins on IL-5 Production of Lymphoid Cells Stimulated with ConA[a]

		mice (pg/mL)				
treatment	lymphocytes	PBS	OVA	Da-dio	Dj-dio	Dp-dio
ConA	spleen	722 ± 153*	218 ± 71	255 ± 192	158 ± 4	255 ± 37
	bone marrow	247 ± 120	238 ± 121	86 ± 10	71 ± 3	510 ± 8*
	thymus	288 ± 64	203 ± 80	68 ± 2	76 ± 10	83 ± 7

[a]Lymphoid cells of the treated mice were stimulated with ConA, and the levels of IL-5 were determined using ELISA assay. The difference between the results of indicated groups (PBS or dioscorin-treated mice) and the positive control (OVA-sensitized mice) was determined by the Student's *t* test, and a *P* value of less than 0.05 was considered to be statistically significant (*).

increased to 1369 μg/mL, 2.5-fold the IgG1 level in the positive control mice.

Effects of Dioscorins on the Production of IFN-γ, IL-2 and IL-5 of Lymphoid Cells. To elucidate the effects of dioscorins on the production of Th1 or Th2 cytokines by the lymphoid cells of OVA-sensitized mice, we determined the levels of IFN-γ, IL-2, and IL-5 secreted by the lymphoid cells of OVA-sensitized mice after ip injection with various dioscorins. We isolated the lymphoid cells from the negative control, positive control, and dioscorin-treated mice, restimulated the lymphoid cells with ConA, and then measured the IFN-γ, IL-2, and IL-5 levels produced by the lymphoid cells. The results in Table 1 reveal that the IFN-γ levels produced by the spleen cells of the dioscorin-treated mice in response to ConA were 2- to 3-fold higher than those of the corresponding positive control mice. However, the levels of IFN-γ produced by the bone marrow cells of the dioscorin-treated mice in response to ConA re-stimulation were comparable to those of the corresponding positive control mice. The levels of IFN-γ in the thymus of dioscorin-treated mice in response to ConA were comparable or slightly lower than those of the positive control mice.

The levels of IL-2 produced by all the lymphoid cells of the dioscorin-treated mice in response to ConA re-stimulation were slightly lower than those of the corresponding positive control mice (Table 2), except for the levels of IL-2 produced by the thymus cells of the Dj-dioscorin-treated and Dp-dioscorin-treated mice, which were 1.3- to 1.5-fold higher than those of the positive control mice.

The levels of IL-5 produced by all the lymphoid cells of the dioscorin-treated mice in response to ConA re-stimulation were noticeably lower than those of the corresponding positive control mice, except for the IL-5 levels produced by the spleen cells of the Da-dioscorin or Dp-dioscorin-treated mice, which were slightly higher than those of the positive control mice. Similarly, the IL-5 levels produced by the bone marrow cells of the Dp-dioscorin-treated mice were more than 2-fold higher than those of the positive control mice (Table 3).

Effects of Dioscorins on the Proliferation of Spleen Cells. To determine whether the lymphoid cells of the dioscorin-treated mice were anergic toward dioscorins or mitogen, we treated the lymphoid cells of the dioscorin-treated mice with OVA, ConA, or various dioscorins for 2 days, and we determined the proliferation of the lymphoid cells. The results revealed that the spleen and bone marrow cells isolated from all the dioscorin-treated mice proliferated in response to ConA but not to OVA or dioscorins (Figure 5A,B). The thymus cells of all the dioscorin-treated mice did not proliferate in response to ConA, OVA, or dioscorins (Figure 5C).

DISCUSSION

Type I allergic responses involve IgE-dependent mast-cell and basophil degranulation and eosinophil accumulation in sites of inflammation.[3] Therefore, regulating IgE production and histamine release is of clinical importance. In this study, we demonstrated for the first time that the major storage proteins of yams, dioscorins, exhibited an immunomodulatory activity for suppressing allergic reactions. Intraperitoneal injection of native dioscorins into OVA-sensitized mice significantly

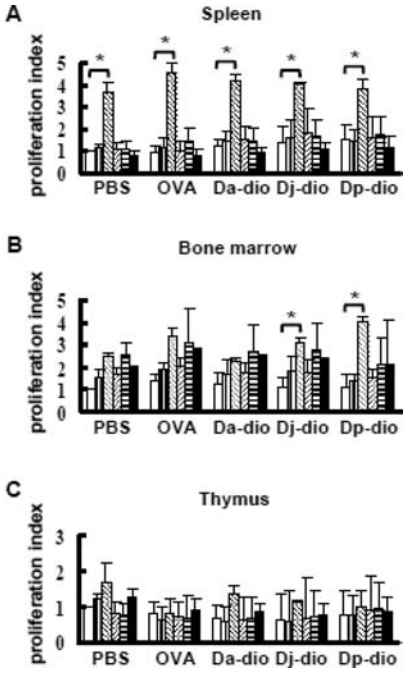

Figure 5. Effects of various dioscorins on the proliferation of lymphoid cells of dioscorin-treated mice. The lymphoid cells isolated from the negative control, positive control, and dioscorin-treated mice were restimulated with PBS (□, OVA (◨), ConA (▧), Da-dioscorins (◪), Dj-dioscorin(⬓), or Dp-dioscorins (■) for 48 h. Proliferation of cells was determined by MTS assay. Three replicates for each treatment were performed. The proliferation index was obtained as compared to that of the negative control restimulated with PBS. Results represent mean ± SD of five mice (n = 5). Asterisk (*) represents a significant difference at $p < 0.05$ of the proliferation indexes between the re-stimulation treatments and that restimulated with PBS of the same group.

decreased serum IgE and histamine levels (Figure 2). The decrease of IgE and histamine levels was concomitant with the significant increase of IFN-γ and IgG2a levels in sera (Figures 3A and 4A), suggesting that dioscorins suppressed the allergic reaction by promoting the Th1 cell response. Moreover, we observed that ip injection of native dioscorins into OVA-sensitized mice decreased the IL-5 levels to basal levels in Da-dioscorin- and Dj-dioscorin-treated mice (Figure 3B), suggesting that dioscorins suppressed the allergic reaction by down-regulating the Th2 cell response. Similar results were observed for the lymphoid cells of the dioscorin-treated mice (Tables 1 and 3). We found that the IFN-γ level in the spleen cells of the dioscorin-treated mice had increased dramatically in response to ConA treatment, when compared with the positive control mice (Table 1). By contrast, the IL-5 level decreased in most of the lymphoid cells treated with ConA (Table 3). These results suggested that the dioscorins suppressed the allergic reaction through modulation of the Th1/Th2 imbalance by promoting the Th1 cell response.

Th1 cells modulate cellular immunity by producing IL-2 and IFN-γ. IFN-γ promotes isotype switching to IgG2a but suppresses switching to IgG1.[7,8] However, we found that although the increase in the IgG2a levels was concomitant with the increase in the IFN-γ levels in all the dioscorin-treated mice, the IgG1 levels did not decrease, and even increased in the sera of the Dp-dioscorin-treated mice (Figures 3A and 4). The suppression of an allergic reaction by down-regulation of the Th2 cell response, but without a concomitant decrease in the IgG1 levels, has been reported for many herbal medicines. Hsu et al. (2003) demonstrated that dehulled adlay had a modulating ability to shift the balance from Th2 to Th1 dominance in OVA-sensitized mice.[27] Oral administration of dehulled adlay decreased the serum IgE and IL-5 levels but increased both IgG1 and IgG2a levels in dehulled adlay-treated mice. Sy et al. (2006) reported that propolis extracts may not only enhance the Th1 cell response but may also inhibit the Th2 cell response.[28] Oral administration of a high dose of propolis extracts to mice for 16 weeks suppressed the serum IgE level but significantly increased both the IgG1 level and IgG2a level. Lin et al. (2006) found that the ip injection of polysaccharides purified from *Ganoderma lucidum* promoted the Th1 cell response in OVA-sensitized BALB/c mice.[26] IgG2a production significantly increased in response to the polysaccharide treatment, whereas IgG1 levels showed no differences between the polysaccharide-treated and the OVA-sensitized mice.

Furthermore, we observed that the IL-5 and IgG1 levels in Da-dioscorin- and Dj-dioscorin-treated mice were either lower or comparable to those of the positive control mice, whereas both IL-5 and IgG1 levels in the sera of Dp-dioscorin-treated mice increased significantly (Figures 3B and 4B). We inferred that the different immunomodulatory effects of dioscorins on OVA-sensitized mice might be due to immune deviation.[5,29] The production of IgE in mice with an immune deviation decreased, although the production of IgG1 and IgG2a increased. The mice with an immune deviation appeared to induce the development of T-cell hyporesponsiveness associated with the development of T_R cells. Alternatively, these results might be due to the various isoforms of native dioscorins in the yam tubers of several species.[15] We considered that the later scenario was more likely, because when we used the recombinant Class A isoform of Dp-dioscorins isolated from *E. coli* to treat the OVA-sensitized mice, the IL-5 and IgG1 levels decreased to values comparable to those of the Da-dioscorin- and Dj-dioscorin-treated mice (unpublished data). Our previous study showed that although dioscorins from different yam species had similar biochemical activities, they exhibited different immunomodulatory effects.[23] Both Da-dioscorins and Dj-dioscorins induced the expression of the pro-inflammatory cytokines and stimulated phagocytosis of the macrophage RAW264.7, whereas Da-dioscorins had a higher stimulation effect than did Dj-dioscorins for the phagocytosis of bone marrow, spleen, and thymic cells. By contrast, Dj-dioscorins enhanced the proliferation of $CD4^+$, $CD8^+$, and $Tim3^+$ (Th1) cells in the spleen and the proliferation of $CD19^+$ cells in both the spleen and the thymus. We recently reported that Da-dioscorins isolated from the yam tuber of *D. alata* were Class A dioscorins, whereas the Dj-dioscorins isolated from the yam tuber of *D. japonica* were mainly Class A, with minor Class B, dioscorins.[15] By contrast, the Dp-dioscorins isolated from the yam tuber of *D. pseudojaponica* consisted of a mixture of Class A and Class B dioscorins. Class A dioscorins are monomers containing one intramolecular disulfide bond between Cys^{28}–Cys^{187}, whereas Class B dioscorins are dimers containing one intermolecular disulfide bond between Cys^{40}–Cys^{40} in addition to the intramolecular disulfide bond.[14,15]

Furthermore, dioscorins from various yam species or cultivars exhibit different levels of glycosylation. Dioscorins extracted from the yam tubers of *D. ratundata* are not glycoproteins,[13] whereas those from the yam tubers of *D.*

cayenensis and *D. batatas* are glycoproteins.[17] Da-dioscorins, Dj-dioscorins, and Dp-dioscorins are thought to be post-translationally modified by glycosylation and/or phosphorylation. This suggestion is based on the observed differences in their molecular weight calculated from the primary structures as well as those interpolated from SDS-PAGE, and this is also based on the charge heterogeneity of the dioscorin isoforms determined by isoelectric focusing gel electrophoresis.[15] Although the extent of post-translational modification of these isoforms has not been determined, the slightly different immunomodulatory effects of dioscorins from various yam species might be due to the different composition of the dioscorin isoforms and/or to the different extent of post-translational modification of the dioscorin isoforms in yam tubers. A recent study showed that the post-translational modification of different isoforms may have different regulatory effects. Two thyroid hormone receptor (TR) isoforms, TRα1 and TRβ1, differentially mediated thyroid hormone action at different target tissues.[30] Post-translational modification of TR by conjugation of small ubiquitin-like modifiers (SUMO) to produce TRα1 and TRβ1 played a crucial role in thyroid hormone action and TR isoform specificity. TRα1 was sumoylated at lysines 283 and 389, and TRβ1, at lysines 50, 146, and 443. Therefore, the effect of dioscorin isoforms and the extent of post-translational modification on immunomodulatory responses merit further investigation.

We observed that the IFN-γ level in the spleen cells of the dioscorin-treated mice dramatically increased in response to ConA treatment, when compared with the positive control mice, whereas the IL-5 level decreased in most of the lymphoid cells treated with ConA, except for the bone marrow cells of the Dp-treated mice (Tables 1 and 3). These results coincided with the production of IFN-γ and the IL-5 levels in the sera of the OVA-sensitized BALB/c mice in response to dioscorin treatment (Figure 3). These results support the idea that dioscorins suppressed the allergic reaction of OVA-sensitized mice through the regulation of Th1/Th2 type cell response. Nevertheless, we found that the level of IL-2 (a Th2 cytokine) in all the lymphoid cells slightly decreased in response to ConA treatment, except for the thymus cells of Dj-dioscorin- and Dp-dioscorin-treated mice (Table 2). It was unclear why the IL-2 level did not increase concomitant with the increase in the IFN-γ level. A similar result was reported by Liu et al. (2009), who found that the oral administration of Da-dioscorins to BALB/c mice for 21 days stimulated the secretion of IFN-γ, IL-4, and IL-10 by spleen cells, whereas the production of IL-2 decreased slightly.[24] The level of IL-2 in the spleen cells of mice that were administered propolis extract in response to ConA stimulation was slightly lower than that of the OVA-sensitized mice.[28] Further research is needed to elucidate why the IL-2 level did not increase concomitantly with the increase in the IFN-γ level in the spleen cells of the dioscorin-treated mice in response to ConA treatment.

Figure 5 shows that the spleen and bone marrow cells of all the dioscorin-treated mice significantly proliferated in response to ConA but not to OVA or dioscorins. The lack of a stimulating effect of the dioscorins on the lymphoid cells suggested tolerance (hyporesponsiveness) or an anergy effect toward dioscorin re-stimulation. Dioscorins improved tolerance to allergen challenge and prevented the development of new allergic sensitization, suggesting that dioscorins could be used for immunotherapy of food allergies. In conclusion, the results of this study indicated that dioscorins suppressed the allergic reactions in OVA-sensitized BALB/c mice, possibly through modulation of the Th1/Th2 imbalance by promoting the Th1 cell response, and could be a potential therapeutic agent against food allergies.

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Notes

The authors declare no competing financial interest.

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