Aqueous Extract of Peanut Skin and Its Main Constituent Procyanidin A1 Suppress Serum IgE and IgG1 Levels in Mice-Immunized with Ovalbumin

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Allergy is a common health problem in developed countries. Most allergic reactions are mediated by allergen-specific IgE antibodies. Therefore, development of a method for inhibiting allergen-specific IgE production is a useful approach to prevent food-allergic diseases. IgE synthesis is considered to be caused by development and activation of T helper-2 (Th2) cells and B cells. This specific Th2 cell produces predominantly interleukin (IL)-4 and IL-5. IL-4 plays a crucial role in inducing class switching of IgM into IgE isotype and the IgE production. IL-5 enhances IL-4-dependent IgE production. In contrast, the T helper 1 (Th1) cells mainly secrete cytokines such as IL-2 and interferon (IFN)-γ that inhibit IgE and IgG1 secretion and enhance IgG2a secretion. Thus, shifting the balance from Th2 to Th1 dominance should be a rational strategy to prevent IgE-mediated allergic diseases.

The skins of mature peanuts (Arachis hypogaea L.) are particularly rich in procyanidins. They primarily contain A type procyanidins, in which the subunits are connected by 4β→8 carbon bonds and 2β→O7 ether bonds to form the A1 dimer and trimer. Peanut skins are used to treat chronic haemorrhage and bronchitis in Chinese traditional medicine. Six A-type proanthocyanidins and flavonoids have been isolated from the water-soluble phenolic fraction from the skin of the mature seed of the peanut, Arachis hypogaea. In this study, we investigated the effect of oral administration of aqueous extract from peanut skin on allergic mediators production in mice sensitized with ovalbumin (OVA).

MATERIALS AND METHODS

Mice Female 8—10-week-old BALB/c mice weighing between 20 to 21 g were purchased from the Japan SLC (Shizuoka, Japan). Mice were housed in groups of five animals in plastic cages with a 12 h light and 12 h dark cycle and free access to water and food. An adaptation to these conditions of at least 1 week was allowed before the start of the experiment (body weights: 22 to 23 g). The experimental procedures complied with the Council for Experimental Animals at the Faculty of Pharmaceutical Sciences, Kanazawa University. Mice were sacrificed by an overdose of ether.

Test Sample Preparation Apple polyphenols and grape seed polyphenol were kindly provided by Dr. Tomoya Takahashi (Kyowa Hakko Kogyo Co., Chiyoda, Tokyo, Japan).

Apple Polyphenols (AP) AP was obtained from unripe apples according to the method described in a previous report. Malus pumila MILLER var. domestica SCHNIEIDER, Fuji variety (Rosaceae) (5.86 kg) was taxonomically identified and deposited in a database at Kyowa Hakko Kogyo Co., under registration number KH-2A. Unripe apples were homogenized in 0.1% potassium pyrosulfite solution. The homogenate was allowed to stand for 24 h at 4 °C and then centrifuged at 3500×g for 30 min at 4 °C. Supernatant was filtered through a glass filter, and the filtrate was passed through a column (5 cm×50 cm) filled with Sepabeads SP-850 resin (Mitsubishi Kasei, Tokyo, Japan), equilibrated previous step with demineralized water, and then washed with 2 l of demineralized water. The column is then eluted with 2 l of 65% (v/v) aqueous ethanol, and the eluted fraction was evaporated under reduced pressure to give 34.3 g of extract. This extract was dissolved in demineralized water and passed...
through a column (5 cm × 50 cm) filled with Diaion HP-20 (Mitsubishi Kasei), equilibrated previously with demineralized water and equal volume of 15% (v/v) aqueous methanol. The column was then eluted with 21 of 45% (v/v) aqueous methanol. The eluted fraction was evaporated to give light-brownish powder (20.6 g), which was then dissolved in 200 ml of methyl acetate, stirred for 1 h at room temperature, and then the solution was filtered. The residue was re-extracted with methyl acetate under the same conditions. Both filtrates were combined and evaporated to give AP (12.0 g).

The total procyanidin content was colorimetrically measured by a method described by Porter et al., and was calculated to be 83.6% (w/w) using procyanidin B-2 as a standard. AP contained 7.3% (w/w) procyanidin B-1 (Fig. 1), 26.2% (w/w) procyanidin B-2 (Fig. 1), and 7.7% (w/w) procyanidin C-1 (Fig. 1) as the major components; other oligomeric procyanidins were also present about 40% (w/w).

**Grape Seed Polyphenols (GP)**

GP was also prepared by Dr. T. Takahashi. In brief, *Vitis vinifera* L. (Vitaceae) was taxonomically identified and deposited in a database at Kyowa Hakko Kogyo Co., under registration number A-2002-1. Raw peanuts (with seed skin, 4.0 kg) were extracted twice with distilled water (6.0 l), each time for 20 min at room temperature. Two samples were combined and lyophilized to give a brownish powder (25.0 g: PSE). Short time maceration of raw peanuts (with seed skin) in distilled water effectively extracted PSE from peanuts skin.

**Purification of Procyanidin A1 (PA) from PSE**

PSE (20 g) was adsorbed on Diaion HP-20 resin (1.0 l), and components were successively eluted with water (21), aqueous acetone (10%: 21, 30%: 21, 50%: 21, 70% 21), and 80% ethanol. The eluate with 80% ethanol (21) was concentrated to dryness under reduced pressure. To remove sugar and high molecular constituents from the eluate, the residue was dissolved in 95% (v/v) ethanol (21) and the solvent was filtered. After evaporation of the solvent under reduced pressure, the extract (12 g) was further fractionated by chromatography over Toyopearl HW 40 (Tosoh Bioscience LLC, Tokyo, Japan, bed volume: 500 ml). Subsequent elution with mixtures of aqueous ethanol and acetone yielded 12 fractions. Fractions 9 and 10 were obtained with ethanol–acetone–water (60 : 15 : 25, v/v/v) and 50% (v/v) acetone respectively, and contained mostly phenolic components as detected by TLC (Merck silica gel 60F254). Fraction 9 (1 g) was subjected to further chromatography on Sephadex LH-20 (GE Healthcare, U.K., 5 × 60 cm), and elution was performed with aqueous acetone. The fractions eluted with 30% acetone contained polyphenols with flavanol moieties, as suggested by the characteristic orange colorations with anisaldehyde reagents. These fractions were further purified by repeated chromatography on Wakogel LP60 C18 (Wako Pure Chem., Tokyo, Japan, 3.5 × 20 cm) and elution was performed with 20% (v/v) methanol in water. Final purification was achieved by preparative HPLC on prep-ODS column (InertsilPREP-ODS, GL Sciences Inc, Tokyo, Japan, 3 × 25 cm) with a mixture of MeOH–tetrahydrofuran–water (20 : 1 : 75) as the mobile phase. The compound (75 mg) was finally crystallized as colorless needles from water. Compound I was identified to be procyanidin A-1 (PA) (Fig. 1) by comparing the spectral data with that reported in the literature.

**Test Sample Treatment**

All samples were dissolved in water and injected orally at doses from 10 to 100 mg/kg/d once a day for 21 consecutive days. For the second experiment, the mice-immunized with OVA were intraperitoneally injected with either 1 to 5 mg/kg/d (for 21 consecutive days) dexamethasone (Sigma chemical, St. Louis, MO, U.S.A., dissolved in 1% (v/v) ethanol–saline solution) or 5 to 30 mg/kg/d (for 21 consecutive days) indomethacin (Sigma, dissolved in 1% (v/v) ethanol–saline solution). Disease control group received vehicle instead of test sample on the same regimen.
Experimental Design Mice were injected intraperitoneally with 20 mg/mouse OVA (albumin from chicken egg white, grade VII, Sigma) with an alum (Al(OH)3) adjuvant (Sigma). Ten days after the first OVA-immunization, mice received boosters using the same dose of OVA. Mice were killed by over-dose of ether 7 d after the second immunization.

Measurement of White Blood Cell (WBC) Numbers in Peripheral Blood Samples Peripheral blood (20 μl/mouse) was collected after 21 d by the retro-orbital venous plexus sampling method with an EDTA-coated capillary tube. WBC count in each sample was counted using an automatic hemocytometer (Horiba, MICROS abc, LC-152, Kyoto, Japan).

Measurement of Serum IgG1 and Cytokine Production To measure levels of immunoglobulin and cytokine in serum, blood samples were collected by intraventricular exsanguination at 21 d after the first sensitization with OVA. Serum was separated by centrifugation after clotting and stored at −80 °C until use. The IL-4 and IFN-γ levels in the serum were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Cyto-SCREENTM, BIOSOURCE, CA, U.S.A.). Serum IgG1 was determined using rat-mouse IgG1 (BD PharMingen, CA, U.S.A.), IgG1 standard (BD PharMingen) and anti-mouse IgG1-horseradish peroxidase (HRP, BD PharMingen). Diluted serum was incubated in duplicate overnight, washed with phosphate buffer-saline containing 0.05% w/v Tween 20 (Wako Pure Chem., Tokyo) (PBS-T), incubated with anti-mouse avidin conjugate IgG1 (BD PharMingen) for 2 h, and then washed several times with PBS-T. Assays were developed with 3,3′,5,5′-tetramethylbenzidine (TMB, BD PharMingen) as a substrate regent, reactions were stopped with 1 N H2PO4 and read at 450 nm using a micro plate reader.

Measurement of Serum OVA-Specific IgE Blood collected after sacrifice on day 21 was immediately centrifuged and serum was collected and stored at −80 °C. ELISA for OVA-specific IgE was measured according to the manufacturer’s recommendations using the rat anti-mouse OVA-specific IgE ELISA assay kit (Dainippon Sumitomo, Tokyo, Japan).

Toxicological Study To investigate the toxicities of PSE, GP, AP and PA by oral administration, body weight of mice in these regimens was checked every day and the activity of hepatic marker enzyme, alanine aminotransferase (ALT, EC 2.6.1.2), in plasma collected on day 21 was also measured using a commercial diagnostic assay kit (Transaminase C-II test Wako, Wako Pure Chem., Tokyo, Japan).

Data Analysis Data were analyzed using statistical analysis and graphing software (Kaleida Graph TM, Synergy Software, Tokyo, Japan). All data are expressed as mean ± S.E. Statistical significance was determined by Dunnett’s multiple test after one-way analysis of variance (ANOVA) with comparison to a disease or normal control group, and the differences were considered significant if p < 0.05.

RESULTS

We first investigated the effect of PSE on spleen weight and peripheral WBC counts in mice-sensitized with OVA. As shown in Fig. 2, intraperitoneal injection of OVA markedly increased spleen weight and WBC counts on day 21 (Figs. 2A, C). PSE injected orally at doses of 1 to 100 mg/kg/d for 21 consecutive days significantly suppressed the increase of spleen weight and the number of circulating WBCs on day 21 (Figs. 2A, C). Administration of PA (1 to 10 mg/kg/d) isolated from PSE also resulted in reduced spleen weight and WBC number, while apple polyphenols (AP) and grape seed polyphenols (GP) at dosages of 30 mg/kg/d was weakly affected the spleen weight and the WBC counts (Figs. 2A, C). Intraperitoneal injection with dexamethasone (1 and 5 mg/kg/d), a typical steroidal anti-inflammatory agent, strongly suppressed WBC counts and swelling of the spleen but indomethacin, a cyclooxygenase inhibitor, did not affect hyperleukocytosis and swelling of the spleen even at higher dose (30 mg/kg/d) (Figs. 2B, D).

In this experiment, oral injection of PSE, GP, AP and PA showed no toxicity in mice, demonstrated by the fact that the body and liver weights (data not shown) and level of plasma hepatic marker enzyme, ALT, did not change (100 mg/kg/d PSE: 22.1 ± 1.0 U/ml, n = 7, not significant; 30 mg/kg/d AP: 26.1 ± 2.9 U/ml, n = 6, not significant; 30 mg/kg/d GP: 19.0 ± 0.4 U/ml, not significant; 10 mg/kg/d PA, 20.4 ± 1.8 U/ml, n = 5, not significant; vehicle only: 18.3 ± 1.1 U/ml, n = 3).

On day 21, blood samples were collected to measure OVA-specific IgE and total IgG1 levels. OVA-specific IgE was not detected in normal mice, while the level was dramatically increased in OVA-immunized mice; the averages of the level in
Day 21. As shown in Fig. 3A, a tendency for IFN-γ levels to decrease was observed in mice-immunized with OVA compared with the normal level of IFN-γ. Fig. 4A shows the cytokines (IL-4) in serum of mice-immunized with OVA. IL-4 levels in serum were dramatically and significantly elevated in control mice (Figs. 3C, D). These test samples did not reverse IFN-γ levels, while indomethacin did not (Figs. 4B, D).

DISCUSSION

The present study demonstrated that oral injection of the aqueous extract of peanut (Arachis hypogaea L.) skin and PA isolated from PSE decreased serum IgE and IgG1 levels as well as WBC counts in mice-immunized with OVA. These indicated PSE had an anti-allergic effect in vivo, and PA was one of the active constituents responsible for the effect of PSE. Hot water extracts of peanut skin have been reported to scavenge oxygen radical species. To our knowledge, the present results provide the first evidence that oral injection of PSE and PA decrease the level of allergic mediators such as IgG1 and IgE.

The skins of mature peanut, apple and grape seed are particularly abundant in procyanidins. Peanut skin primarily contains A type procyanidins, in which the subunits are connected by both 4β→8 carbon bonds and 2β→O7 ether bonds as shown in Fig. 1 for dimer A1 (Fig. 1). In contrast, procyanidins from apple and grape seed are primarily composed of types B and C, in which the monomers are linked by 4β→8 bonds for the B2 dimer and C trimer (Fig. 1), respectively. We therefore tried to compare the effects of GP and AP with the effects of PSE on IgE and IgG1 levels in mice-immunized with OVA. Orally administered AP at a
dose of 30 mg/kg/d significantly reduced IgE levels, whereas AP and GP did not reduce total IgG1 levels at the same dose (Figs. 3A, C). Identical results were obtained when the mice were injected orally with these polyphenols even at higher doses (100 mg/kg/d; data not shown). Thus, it could be assumed that the anti-allergic activities of these three different extracts depend on their procyanidin contents. Further purification and chemical characterization of the active constituents from PSE, GP and AP as well as evaluation of their anti-allergic effects are necessary.

The balance between Th1 and Th2 is of central importance for systemic immune responses.17 It is also indicated that overexpression of Th2 and/or Th1-depolarization predispose for allergic diseases such as allergic asthma.28 We therefore measured serum cytokine levels, IFN-γ and IL-2, in correlation with Th1/Th2 polarization in mice orally injected with polyphenol samples after OVA immunization.

In this experiment, all tested polyphenols, PSE, PA, GP and AP were found to significantly decrease the enhancement of IL-4 levels induced by OVA (Fig. 4C), whereas only PSE and PA could reverse IFN-γ levels reduced by OVA (Fig. 4A). It is well known that Th2 cells, which produce IL-4 (enhancing IgE synthesis), IL-5 (increasing eosinophil growth and differentiation), and IL-13 (increasing mucus production and inducing airway hyperreactivity) up-regulate and amplify the allergic inflammatory response.25—27 On the other hand, Th1 cells, which produce IL-12 (increasing IFN-γ production) and IFN-γ (suppressing differentiation of naive T cells to Th2 subtypes and local recruitment of eosinophils) are capable of counteracting Th2 responses and vice versa.28—30 We previously reported that intestinal lymphoid cells from mice injected orally with culture fluid from entomogenous fungus, Paecilomyces tenuipes, selectively induced Th1 cytokines (IL-2 and IFN-γ) production, and the culture fluid has the potential of a therapeutic or preventive agent for allergy.31 Thus, it is possible to suggest that the mechanism by which PSE and PA decrease the levels of both IgE and total IgG1 is in part due to an induction of Th2-depolarization. Furthermore, it is also shown that oral administration of GP and AP reduced the IL-4 level without any significant affect on IFN-γ, which may explain why the suppressive effects of these polyphenols are weaker than those of PSE and PA.

Intrapertioneal administration of dexamethasone and indomethacin, which used as anti-inflammatory positive control drugs, showed opposing effects on the serum allergic mediator and Th cytokine levels in mice-immunized with OVA; dexamethasone decreased the serum immunoglobulin and cytokines levels but indomethacin did not (Figs. 2-4). It is well established that the corticosteroid dexamethasone inhibits inflammatory cytokine production in a murine model of allergic asthma in vivo.29 The beneficial effect of corticosteroids in allergic disease is in part due to their direct inhibitory effects on inflammatory cytokines production via a corticosteroid receptor. In contrast to its efficacy of dexamethasone, indomethacin reported to increase production of IL-5 and IL-13 and airway hyperresponsiveness in OVA-sensitized mice by inhibiting prostaglandins E2 synthesis through the cyclooxygenase pathway.51 It could therefore be discussed that the difference between dexamethasone and indomethacin on anti-inflammatory mechanisms affects the results of immunoglobulin synthesis and cytokine secretion in mice-immunized with OVA.

Taken together, PSE may have the potential to suppress allergic diseases by inhibiting immunoglobulin synthesis and regulating systemic T helper cytokine productions. It was also revealed that PA isolated from PSE might be one of an anti-allergic constituent of PSE. However, the anti-allergic efficacy of PA was similar to PSE or slightly lower than that of PSE, indicating that the activity attributed to PSE might be related to different compounds besides PA. We are now investigating the isolation of other active constituents from PSE (including GP and AP) and the effects of PSE and PA on allergic symptoms and production of chemical mediators in mice-immunized with OVA.

REFERENCES


