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Issue: *Foods for Health in the 21st Century***Effect of walnut (*Juglans regia*) polyphenolic compounds on ovalbumin-specific IgE induction in female BALB/c mice**Sarah S. Comstock,¹ Laurel J. Gershwin,² and Suzanne S. Teuber¹¹Department of Internal Medicine, Division of Rheumatology, Allergy, and Clinical Immunology, University of California, Davis, School of Medicine, Davis, California, USA. ²Department of Pathology, Microbiology, and Immunology, University of California, Davis, School of Veterinary Medicine, Davis, California, USA

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English walnuts are implicated in severe, IgE-mediated food allergy in humans. We sought to determine if polyphenolic compounds extracted from the edible nut could promote IgE production to a coadministered allergen. BALB/c mice were sensitized to ovalbumin (OVA) with or without alum (AL) or polyphenolic-enriched extract via intraperitoneal injection. Serum was analyzed for total IgE and OVA-specific IgE, IgG₁, and IgG_{2a/2b}. Coadministration of walnut polyphenolic-enriched extract with antigen and AL increased serum concentrations of antigen-specific IgE and IgG₁. When AL was excluded from the injections, polyphenolic extract tended to enhance OVA-specific IgE and IgG₁ over levels induced by OVA alone, but the increase did not reach significance. Serum IgG_{2a/2b} levels were similar between mice receiving OVA/AL and OVA/AL with polyphenolics. Thus, walnut polyphenolic extract enhanced the Th2-skewing effect of an aluminum hydroxide adjuvant. This indicates that walnut polyphenolic compounds may play a role in allergic sensitization of genetically predisposed individuals.

Keywords: IgE; food allergy; food matrix; polyphenol; walnut; *Juglans*; mouse; antibodies; allergy; sensitization

Introduction

Tree nuts are one of eight foods responsible for about 90% of allergic reactions to foods in the United States.¹ Unlike cow's milk and egg allergy, wherein the majority of children tolerate the food after a period of avoidance, tree nut allergy appears to be lifelong, though it appears about 9% of patients will become tolerant over time.²

Seed storage proteins are the major tree nut allergens. In walnut these are 2S albumins, legumin-group proteins (11S globulins), and vicilin-like proteins (7S globulins).^{3–7} However, unlike shrimp- or fish-allergic individuals, who tend to have IgE against a single protein, individuals with walnut allergy generate IgE against multiple seed storage proteins. This suggests that factors in the food or gut microenvironment which colocalize with the proteins contribute to Th2 skewing and result in IgE to multiple, concurrently presented food antigens.^{8,9}

These factors could be lipids,^{10,11} lectins,^{12,13} carbohydrates,¹⁴ trypsin inhibitors, or polyphenolic compounds.¹⁵ In fact, others have shown that the adjuvant present at the time of sensitization can cause normally Th1-skewed mouse strains to produce a Th2 profile and vice versa.^{16,17} More recently, it has been shown that components of the food matrix are vital to allergic sensitization.^{10,18} Herein, we look at the Th2-skewing potential of an English walnut polyphenolic-enriched extract using a murine model of Th2 sensitization.

Polyphenolic compounds have not yet been linked to food allergy. However, plant-phenolic compounds from sources other than food have been linked with enhanced IgE production.^{15,19} Additionally, polyphenolic compounds have immunomodulatory effects.²⁰ Finally, phenolic compounds are found complexed with proteins, even allergens,²¹ and complexed proteins are more likely

to be internalized by antigen presenting cells.^{9,22} Thus, it is plausible that the polyphenolic content of a food could influence its allergenicity. We undertook studies in BALB/c mice to determine if administration of English walnut (*Juglans regia*) polyphenolic compounds with an antigenic peptide, ovalbumin (OVA), could change the subsequent serum antibody concentrations and *in vitro* cytokine production by spleen cells isolated from the mice.

Materials and methods

Animals

Six- to 11-week-old, female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA) were housed four per cage and given food (Conventional Lab Chow, Purina, St. Louis, MO, USA; or Basal Diet 5755, Test Diet, Richmond, IN, USA) and water *ad libitum*. Animals were maintained as required under guidelines from the National Institutes of Health. The University of California, Davis, Committee on Animal Use and Care approved the protocol.

Polyphenolic preparations and assay for total phenolics

Walnuts were extracted as previously described.²³ The extract likely contained chemical compounds in addition to polyphenolics but was extensively extracted with methanol followed by hexanes to remove lipids. The extract contains ellagic acid (EA), valoneic acid, pedunculagin, other ellagitannins, as well as a quercetin pentoside.²³ Following a procedure, colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents, published previously,²⁴ the concentration of phenolic compounds in polyphenolic solutions was determined. Concentrations are expressed as gallic acid equivalents (GAE).

Immunizations

Mice received 10 μ g of OVA (chicken egg, grade 5; Sigma-Aldrich, St. Louis, MO, USA) in 2 mg of aluminum hydroxide gel (AL) i.p. Walnut polyphenolics were added at 5 μ M, 5 mM, or 50 mM GAE (5 μ M/OVA/AL, 5 mM/OVA/AL, etc.). Total injected volume was 200 μ L. Negative controls received phosphate buffered saline (PBS) with AL. Positive controls received OVA with AL (OVA/AL).

Some treatment groups received OVA alone or various concentrations of polyphenolics in combination with OVA but no AL (OVA, 5 μ M/OVA, 5 mM/OVA). Mice were sensitized on Day 0, boosted on day 21, and sacrificed on day 30 or day 180. Blood was taken on days 0, 30, 45, 60, 75, 90, and 180 or only on day 30. Sera were stored at -70°C .

Measurement of serum ovalbumin-specific IgE

Ninety-six well plates (CoStar, Cambridge, MA, USA) were coated with rat antimouse IgE mAb (Southern Biotechnologies, Birmingham, AL, USA) at 1 μ g/mL in carbonate/bicarbonate buffer (pH 9.6) by overnight incubation at 4°C . Plates were washed with PBS/0.05% Tween-20 (Bio-Rad, Hercules, CA, USA). After a 2-h incubation with 10% bovine serum albumin (BSA) (Sigma-Aldrich) at room temperature (RT), plates were washed, and diluted samples and standards added. Plates were incubated for 2 h at RT and washed. Biotinylated OVA was added. OVA, of the same type used for the immunizations, was linked to Sulfo-NHS-LC-Biotin using the EZ-Link Sulfo-NHS-LC-Biotin method by Pierce (Rockford, IL, USA). Plates were incubated for 1 h at RT, washed and streptavidin-HRP (S-HRP) (R&D Systems, Minneapolis, MN, USA) was added. Plates were incubated for 1 h and washed before detection with stabilized hydrogen peroxide mixed with stabilized tetramethylbenzidine (TMB) (R&D Systems). After 30 min, 2 N sulfuric acid was added to stop the reaction. Plates were read at 450 nm on a Wallac Victor² 1420 Multilabel Counter (Perkin Elmer, Wellesley, MA, USA). OVA-specific IgE is expressed in arbitrary units and calculated from the linear portion of a standard curve generated from serial dilutions of a pool of mouse serum high in OVA-specific IgE. All sera were examined in duplicate.

Measurement of serum OVA-specific IgG₁ and IgG_{2a/2b}

Microplates (CoStar) were coated with OVA (200 μ g/mL (IgG₁) or 300 μ g/mL (IgG_{2a/2b})) by overnight incubation at 4°C . Plates were washed with PBS/0.05% Tween-20. After a 2-h incubation with 10% BSA at RT, plates were washed, and diluted sera samples and standards were added. Plates were incubated for 2 h at RT and washed. Biotinylated rabbit antimouse IgG₁ (Zymed, S. San Francisco,

CA, USA) or biotinylated rat antimouse IgG_{2a/2b} (BD PharMingen, Franklin Lakes, NJ, USA) was added. Plates were incubated for 1 h at RT, washed, and S-HRP was added. Plates were incubated for 1 h at RT and washed before detection using TMB. After 25 min (IgG₁) or 15 min (IgG_{2a/2b}) of development, 2 N sulfuric acid was added to stop the reaction. Plates were read at 450 nm on the Wallac. OVA-specific IgG₁ and IgG_{2a/2b} are expressed in arbitrary units and calculated from the linear portion of a standard curve generated from serial dilutions of a pool of mouse serum that was high in OVA-specific IgG. All sera were examined in duplicate.

Measurement of total serum IgE

OptEIA Mouse IgE ELISA sets (BD Biosciences, San Diego, CA, USA) and the OptEIA Reagent Set B were used according to the manufacturer's instructions. All sera were examined in duplicate. Plates were read on a PowerWave Select X (Bio-Tek Instruments, Winooski, VT, USA).

Splenocyte isolation and culture

Spleens were removed on day 30 and placed in petri dishes containing RPMI 1640 (Gibco, Carlsbad, CA, USA) with 5% fetal bovine serum (FBS) (Gibco) (RPMI-W). Splenocytes were isolated. Cells were washed in RPMI-W, treated to lyse red blood cells, and washed. Cell viability was determined by Trypan blue (Gibco) exclusion. Cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine (Gibco), and 45 µg/mL gentamycin (Gibco). For each mouse, three wells of 2.5×10^6 splenocytes were plated in 48 well plates (CoStar). Each well included 100 µg OVA/mL. Cells were kept in 5.2% CO₂ at 37°C for 72 h. Plates were spun at 1000 rpm for 5 min and supernatants were transferred to sterile tubes. Supernatants were stored at -70°C until analysis.

Measurement of cytokines from cell culture supernatants and serum

Quantikine M kits (R&D Systems) for IL-4, IL-13, and IFN-γ were used to measure cytokines from cell culture supernatants. Alternatively, Luminex Mouse Th1/Th2 Six-Plex kits (Invitrogen BioSource, Carlsbad, CA, USA) were used to measure cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, and IFN-γ) from cell culture supernatants or mouse serum. Kits were used

as directed by the manufacturers. All samples were analyzed in duplicate.

Statistical analysis

Differences in antibody and cytokine levels were determined by one-way ANOVA followed by Fischer's least significant difference test. To determine the effects of treatments over time, two-way ANOVA followed by Fischer's least significant difference test was done. To test the correlation between antibody levels and cytokine expression, Pearson's correlation was used. Systat 9 by SPSS Inc. (Chicago, IL, USA) was used for all statistical analyses. A *P* value <0.05 was considered significant.

Results

Walnut polyphenolic-enriched extract dissolved in water has no effect on IgE production

Mice were injected with 5 mM walnut polyphenolic-enriched extract in the presence of OVA and AL. Polyphenolic-enriched extracts were mixed with water. Mice treated with walnut polyphenolic-enriched extract tended to produce less OVA-specific IgE as well as less total IgE than those injected with OVA and AL (Fig. 1). However, no significant differences in IgE production were seen between the walnut-treated group and the positive control.

Walnut polyphenolic-enriched extract tends to increase OVA-specific IgE production in the absence of adjuvant

Since few significant differences were seen when the polyphenolic-enriched extracts were diluted in water prior to injection, we next determined if another type of diluent might be more appropriate as many of the walnut polyphenolic compounds are only slightly soluble in water.²⁵ In this experiment, animals were injected with OVA in the presence of PBS, dimethyl sulfoxide (DMSO), or 5 mM walnut polyphenolic-enriched extract dissolved in DMSO. No AL was administered in this experiment. Two groups of walnut-treated mice were included. One group contained mice that were 7 weeks of age and the other contained mice that were 12 weeks of age. Although several mice in each of the walnut-treated groups produced high levels of total IgE, none of the

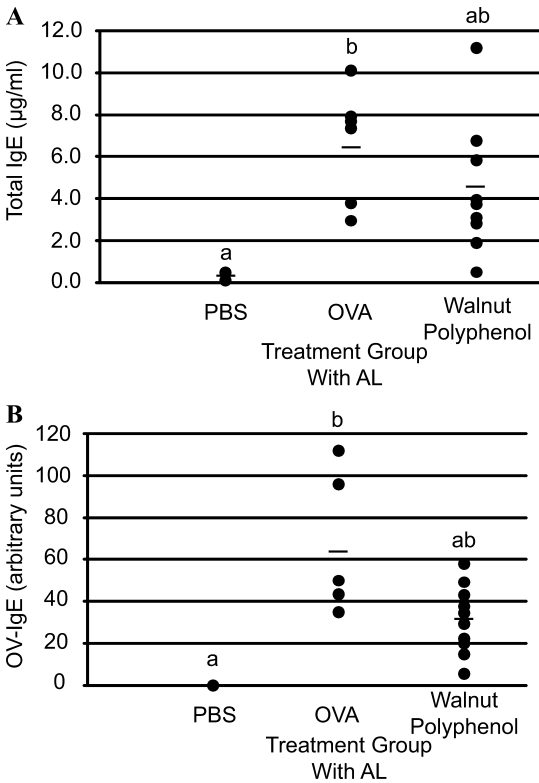


Figure 1. Day 30 total (A) and OVA-specific (B) serum IgE in mice injected with OVA or OVA and walnut polyphenolic-enriched extract. All injections included AL. Polyphenolic-enriched extracts were dissolved in distilled water. Columns bearing a common letter are not significantly different ($P < 0.05$). Lines indicate group means ($n = 7\text{--}8$ mice per group).

treatments produced significantly greater amounts of total IgE when compared to the vehicle control group (DMSO) (Fig. 2A). However, 12-week-old mice injected with walnut polyphenolic-enriched extract dissolved in DMSO showed increased OVA-specific IgE production over that seen when OVA was injected with PBS, but this effect was largely due to the response in a single mouse (Fig. 2B). DMSO itself, when injected in equivalent quantities to those found in the 5 mM walnut polyphenolic injections, did not significantly increase either total or OVA-specific IgE production (Fig. 2).

Time course and persistence of IgE, IgG₁, and IgG_{2a/2b} responses

Because dissolving the walnut polyphenolic-enriched extract in DMSO tended to increase IgE

production in 12-week-old mice, those conditions were used to test the effects of several concentrations of walnut polyphenolic-enriched extract on the induction of a wider variety of antibody isotypes. These effects were followed over a period of 180 days. Female, 12-week-old, BALB/c mice were sensitized to OVA via i.p. injection with AL or AL and walnut polyphenolics at days 0 and 21. Blood was drawn from each mouse at 0, 30, 45, 60, 75, 90, and 180 days after sensitization. OVA-specific IgE, IgG₁, and IgG_{2a/2b} as well as total IgE were determined by ELISA (Fig. 3).

Within 48 h of injection, 7 of 10 mice injected with 50 mM DMSO-dissolved walnut polyphenolic-enriched extract in the presence of AL and OVA died. Two of the three remaining mice died within 5 days

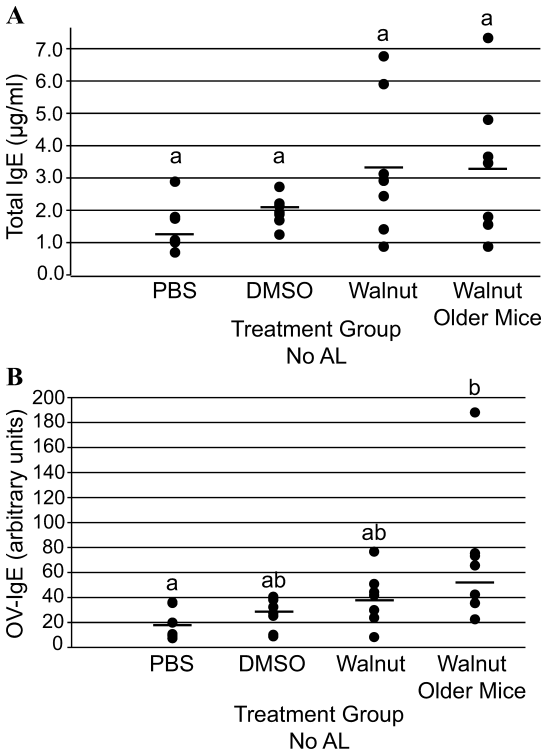


Figure 2. Day 30 total (A) and OVA-specific (B) serum IgE in mice injected with PBS, DMSO, or walnut polyphenolic-enriched extract. All injections included OVA. Polyphenolic-enriched extracts were dissolved in DMSO. Mice were either seven weeks old (Walnut) or 12 weeks old (Walnut Older Mice). Columns bearing a common letter are not significantly different ($P < 0.05$). Lines indicate group means ($n = 6\text{--}8$ mice per group).

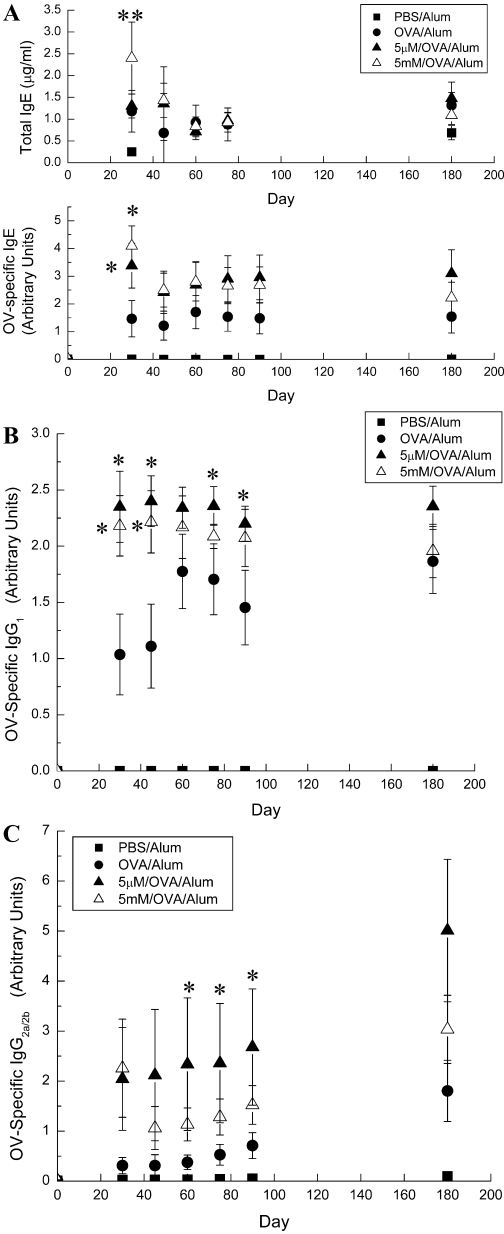


Figure 3. Serum antibody levels in 12-week-old mice injected with PBS, OVA, OVA with 5 μ M walnut polyphenolic-enriched extract, or OVA with 5 mM walnut polyphenolic-enriched extract. All injections included AL. Polyphenolic extract was dissolved in DMSO. Blood samples were collected at days 0, 30, 45, 60, 75, 90, and 180. Isotypes measured included IgE (A), IgG₁ (B), and IgG_{2a/2b} (C). Results are expressed as mean $\pm$ SEM ($n = 10$ mice per group). ** $P < 0.01$ compared with PBS/AL treated mice, * $P < 0.05$ compared with OVA/AL treated mice.

of injection. The remaining mouse was not boosted, and the treatment group was dropped from further studies.

All OVA-specific antibody levels were negligible on day 0 for each treatment group and remained negligible in the PBS/AL control mice over time. Mice treated with either 5 μ M or 5 mM walnut polyphenolics had significantly higher levels of OVA-specific IgE and IgG₁ at day 30 than the OVA/AL group ($P < 0.05$). Serum IgG_{2a/2b} levels also tended to be higher in the mice treated with walnut polyphenolic extract (Fig. 3C). Treatment ($P < 0.001$) but not day ($P = 0.182$) affected serum OVA-specific IgE; however, there was a trend for peaking at day 30 in the polyphenolic-treated mice (Fig. 3A). OVA-specific IgG₁ behaved similarly (treatment, $P < 0.001$; day, $P = 0.874$) (Fig. 3B). On day 30, total IgE levels were elevated only in the 5 mM/OVA/AL group ($P = 0.004$). Total IgE did not differ among the groups past day 45. In all cases, treatment and day did not interact ($P > 0.9$). However, all OVA-specific antibody levels in the walnut polyphenolic-treated mice tended to be higher than those in the OVA/AL mice.

Walnut polyphenolics added to alum augment the day 30 OVA-specific IgE and IgG₁ response

A larger number of 12-week-old BALB/c mice were sensitized to determine if the small increase in OVA-IgE demonstrated in Figure 2B was observed when walnut polyphenolic-enriched extract was coadministered with the Th2 adjuvant AL. On day 30, mice were killed and serum was collected. Total IgE was higher in mice receiving 5 mM/OVA/AL ($4.28 \pm 1.1 \mu\text{g/mL}$, $P < 0.0001$) than in mice receiving OVA/AL ($2.09 \pm 0.53 \mu\text{g/mL}$). Furthermore, serum OVA-specific IgE was higher in mice injected with 5 μ M/OVA/AL (3.14 ± 0.54 , $P = 0.032$) or 5 mM/OVA/AL (3.62 ± 0.51 , $P = 0.003$) than in mice injected with OVA/AL (1.97 ± 0.46) (Fig. 4A). This was also true for OVA-specific IgG₁ where mice injected with 5 μ M/OVA/AL (2.22 ± 0.26 , $P = 0.037$) or 5 mM/OVA/AL (2.31 ± 0.20 , $P = 0.016$) had higher levels than mice injected with OVA/AL (1.64 ± 0.28) (Fig. 4B). On day 30, OVA-specific IgG_{2a/2b} levels did not differ for any treatment groups ($P > 0.05$), and all treatment groups expressed

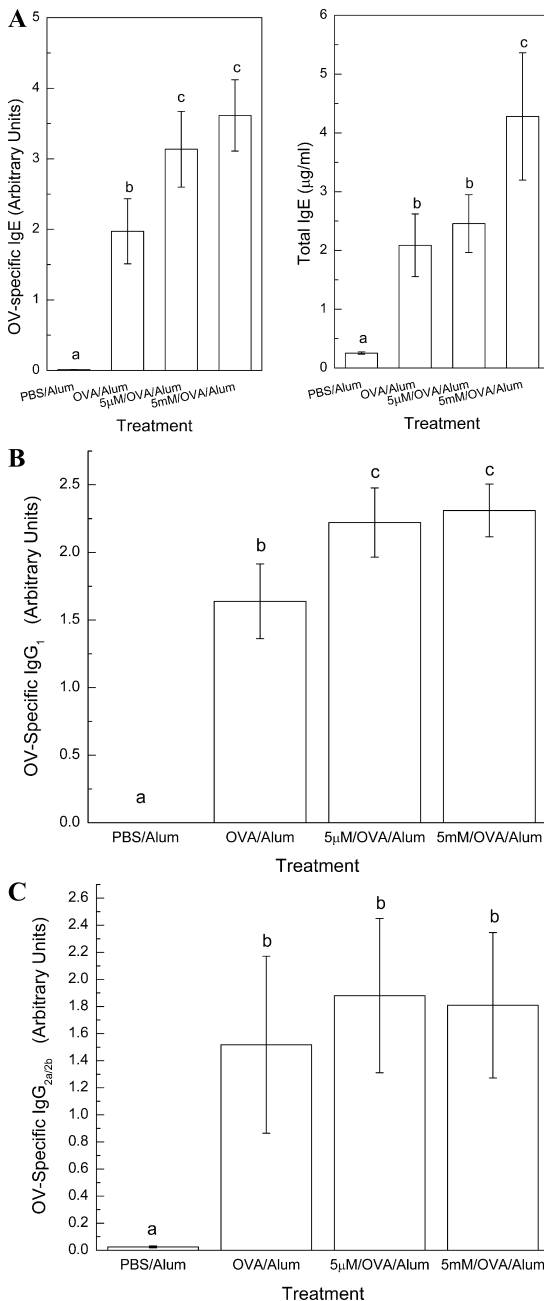


Figure 4. Day 30 serum antibody levels in 12-week-old mice injected with PBS, OVA, and OVA with 5 μ M walnut polyphenolic extract or OVA with 5 mM walnut polyphenolic extract. All injections included AL. Polyphenolic extract was dissolved in DMSO. Isotypes measured included: IgE (A), IgG₁ (B), and IgG_{2a/2b} (C). Results are expressed as mean $\pm$ SEM ($n = 20$ mice per group). Columns bearing a common letter are not significantly different ($P < 0.05$).

higher IgG_{2a/2b} levels than the negative control group ($P < 0.05$) (Fig. 4C).

Walnut polyphenolics at 5 mM tend to increase OVA-specific IgE, IgE, but not IgG_{2a/2b}

To determine if polyphenolics dissolved in DMSO could have an effect on IgE production in the absence of other adjuvants, mice were injected as before; however, AL was excluded from some injections. The addition of 5 mM walnut polyphenolic-enriched extract in the absence of AL tended to increase total IgE production over that seen with administration of OVA alone or with 5 μ M polyphenolics (Fig. 5A). In addition, total IgE ($P = 0.459$) and OVA-specific serum IgE ($P = 0.412$) levels are the same in mice treated with 5 mM/OVA as those in mice treated with OVA/AL. As expected, mice treated with OVA alone have less OVA-specific IgE than mice treated with OVA in the presence of AL ($P = 0.025$). Although mice treated with 5 mM/OVA (1.90 ± 0.028) had higher OVA-specific IgE levels than mice treated with 5 μ M/OVA (0.49 ± 0.16 , $P = 0.050$), the direct comparison of OVA-specific IgE levels between mice treated with OVA alone (0.91 ± 0.23) and those with 5 mM/OVA did not reach significance ($P = 0.162$).

For OVA-specific serum IgG₁ (Fig. 5B), the mice treated with 5 mM/OVA expressed less serum OVA-specific antibody than those treated with OVA/AL ($P = 0.028$), and mice treated with OVA alone have less OVA-specific IgG₁ than those treated with OVA/AL ($P < 0.001$). In the absence of alum, IgG_{2a/2b} antibody levels do not differ from those in the negative control ($P > 0.05$), but OVA/AL administration results in a significant increase in IgG_{2a/2b} antibody ($P < 0.05$) (Fig. 5C).

In summary, addition of 5 mM walnut polyphenolics in the absence of alum tends to increase levels of circulating OVA-specific IgE and IgG₁, but not levels of IgG_{2a/2b}. AL, in addition to increasing levels of circulating OVA-specific IgE and IgG₁, also increases OVA-specific IgG_{2a/2b} levels (Fig. 5).

Measurable cytokines in serum of mice

Once we established that walnut polyphenolics administered in DMSO with AL could increase day 30

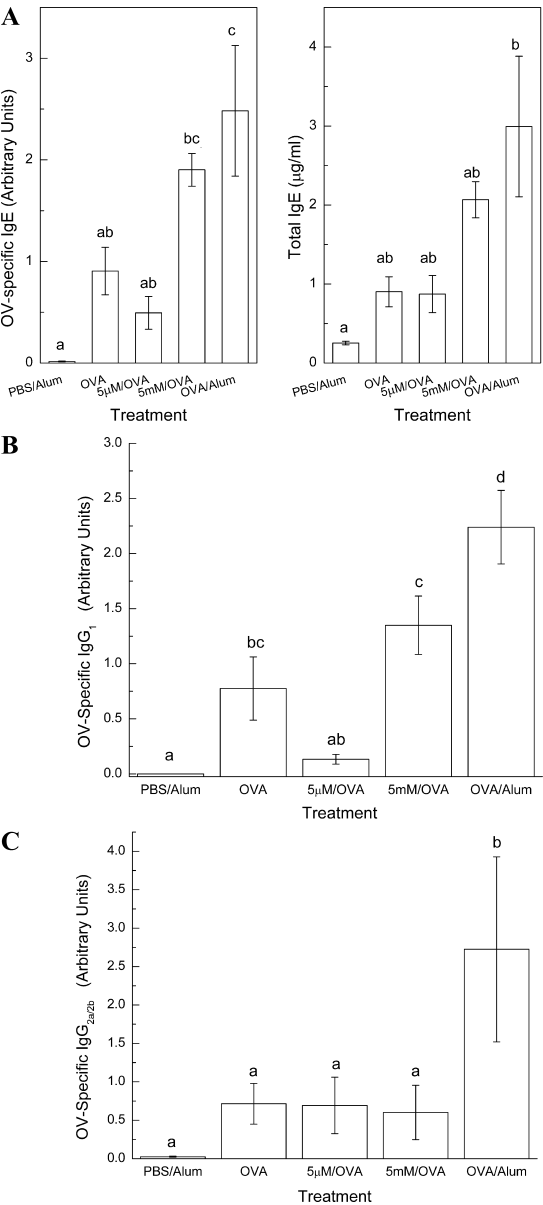


Figure 5. Day 30 serum antibody levels in 12-week-old mice injected with PBS with AL, OVA alone, OVA with AL, OVA with 5 µM walnut polyphenolic extract, or OVA with 5 mM walnut polyphenolic extract. Polyphenolic extract was dissolved in DMSO. Isotypes measured included: IgE (A), IgG₁ (B), and IgG_{2a/2b} (C). Results are expressed as mean ± SEM (*n* = 10 mice per group). Columns bearing a common letter are not significantly different (*P* < 0.05).

OVA-specific IgE and IgG₁, we examined changes in cytokine expression in those animals. The levels of six cytokines were measured in day 30 serum samples. IFN-γ, IL-10, IL-2, and IL-4 could not be detected in serum samples from the mice. No differences were seen in levels of IL-5 (6.13 ± 4.27 pg/mL) or IL-12 (58.79 ± 28.87 pg/mL) (values are average ± SD for all mice combined).

Cytokine expression by splenocytes

To confirm mice expressed a Th2 profile in response to OVA immunization, and to investigate whether splenocytes from mice that had received polyphenolics 9 days prior had a quantitatively higher degree of Th2 cytokine secretion in response to OVA, splenocytes were harvested on day 30 and stimulated in culture. No polyphenolics were added to the culture plates.

Levels of IL-4, IL-13, and IFN-γ were measured by ELISA (Table 1). IL-4 and IL-13 expression in the treatment groups did not differ from that in the OVA/AL positive control group (Table 1). IFN-γ levels for all groups did not differ from that of the negative control (Table 1). When all measurements were combined, IL-4 levels were positively correlated with serum IgE levels (*r* = 0.68).

Because few differences were detected in IL-4, IFN-γ, and IL-13 levels in splenocyte cultures, a Luminex kit (Invitrogen BioSource) was used to measure the expression of IL-2, IL-12, IL-5, and IL-10 in splenocyte cultures from mice injected without AL adjuvant. No differences were seen in levels of IL-2 or IL-10 production for any of the groups tested (Table 1). IL-12 production was lower in all mice immunized with OVA than in the PBS control animals (Table 1). Additionally, IL-5 levels were higher in all mice immunized with OVA than in the PBS control animals (Table 1).

Overall, the increase in IL-4, IL-13, and IL-5 levels in splenocyte cultures from OVA-immunized animals over those seen in cultures from negative control animals are consistent with a Th2 response. The decrease in IL-12 levels in splenocyte cultures from OVA-immunized animals compared to levels in splenocyte cultures from negative control animals is also consistent with a Th2 response.

Table 1. Cytokine expression in splenocyte cultures stimulated with OVA for 72 h

Cytokine	Treatment group	Mean $\pm$ SD (pg/mL)
IL-4	PBS/AL	0 ^a
	OVA	22.88 $\pm$ 29.16 ^b
	OVA/AL	28.11 $\pm$ 27.46 ^b
	5 μ M/OVA	13.77 $\pm$ 13.47 ^{ab}
	5 μ M/OVA/AL	22.95 $\pm$ 20.30 ^b
	5 mM/OVA	30.12 $\pm$ 18.65 ^b
	5 mM/OVA/AL	32.55 $\pm$ 25.25 ^b
IL-13	PBS/AL	43.44 $\pm$ 28.15 ^a
	OVA	1215.23 $\pm$ 600.89 ^b
	OVA/AL	1246.76 $\pm$ 459.54 ^b
	5 μ M/OVA	830.11 $\pm$ 486.33 ^b
	5 μ M/OVA/AL	650.35 $\pm$ 334.94 ^{ab}
	5 mM/OVA	967.39 $\pm$ 451.67 ^b
	5 mM/OVA/AL	832.23 $\pm$ 504.20 ^b
IFN- γ	PBS/AL	8592.74 $\pm$ 7072.83 ^a
	OVA	11817.17 $\pm$ 5150.56 ^a
	OVA/AL	12002.34 $\pm$ 3623.52 ^a
	5 μ M/OVA	8675.12 $\pm$ 4140.69 ^a
	5 μ M/OVA/AL	9154.95 $\pm$ 4941.16 ^a
	5 mM/OVA	8843.75 $\pm$ 5864.06 ^a
	5 mM/OVA/AL	15365.54 $\pm$ 4295.11 ^a
IL-2	PBS	129.06 $\pm$ 12.21 ^a
	OVA	135.82 $\pm$ 9.61 ^a
	5 μ M/OVA	126.99 $\pm$ 19.71 ^a
	5 mM/OVA	124.46 $\pm$ 26.50 ^a
IL-12	PBS	54.73 $\pm$ 15.01 ^a
	OVA	37.87 $\pm$ 10.02 ^b
	5 μ M/OVA	39.35 $\pm$ 3.52 ^b
	5 mM/OVA	38.45 $\pm$ 11.31 ^b
IL-5	PBS	10.93 $\pm$ 5.68 ^a
	OVA	538.74 $\pm$ 447.84 ^b
	5 μ M/OVA	261.58 $\pm$ 191.33 ^b
	5 mM/OVA	255.55 $\pm$ 160.67 ^b
IL-10	PBS	44.72 $\pm$ 11.80 ^a
	OVA	59.78 $\pm$ 16.40 ^a
	5 μ M/OVA	46.46 $\pm$ 9.09 ^a
	5 mM/OVA	53.72 $\pm$ 21.79 ^a

Note: Within a cytokine, measures bearing a common letter are not different ($P < 0.05$).

Discussion

Results of these studies show that walnut polyphenolic-enriched extract can enhance Th2 an-

tibody production (Fig. 4). The effects of adding walnut polyphenolic-enriched extract at sensitization tended to persist over the course of 180 days (Fig. 3). However, enhanced Th2 antibody production was dependent upon the age of the animal, the solution in which the extract was administered and the presence of a Th2-skewing adjuvant (AL). Administration of walnut polyphenolic-enriched extract in the absence of a Th2-skewing adjuvant tended to increase Th2 antibody production over levels seen when OVA alone was administered, but the differences did not reach significance (Fig. 5). Such treatment did not increase OVA-specific IgG_{2a/2b}. However, administration of AL increased levels of Th2 (IgE and IgG₁) and Th1 (IgG_{2a/2b}) antibodies. Administration of the polyphenolic-enriched extract did not induce long-lasting effects on antigen-specific cytokine production by washed splenocytes, obtained 9 days after the last polyphenolic exposure (Table 1). Polyphenolic administration also did not result in any measurable differences in circulating cytokine levels at day 30. Finally, the effects on serum total IgE and OVA-specific IgE concentrations were significant only in the short-term (day 30) (Fig. 3).

Plants synthesize polyphenolics in response to environmental insults and also use them as signaling molecules. In tree nuts, polyphenolic compounds are concentrated in the “skin” or pellicle of the nut but are also found in the kernel.²⁶ EA, a hydrolysable tannin, is the major polyphenolic compound in the pellicle of English walnuts; gallic acid, chlorogenic acid, and juglone, among others, are also present.^{26–28} Other tree nuts also contain EA.^{29,30} The increased antigen-specific IgE and IgG₁ production we saw is in agreement with that seen by others who sensitized mice in the presence of polyphenolic compounds (including chlorogenic acid, a compound found in walnuts) from tobacco.^{15,19} Additional studies have shown that polyphenolic compounds skew toward a Th2 immune response.^{29,31–36}

Though the mechanism for the increased antigen-specific IgE in our animals is unknown, several possibilities exist. The phenolic compounds may exert their effects through modulation of cytokine expression by immune cells. In general, murine B cells that have cross-linked CD40 molecules on their surfaces, are induced to switch to IgE production by the presence of IL-4 or IL-13 in the microenvironment.^{37–42} Additionally, although IL-5 cannot increase IgE

production alone, IL-5 has been shown to potentiate the IgE response in situations where IL-4 levels are suboptimal.⁴³ It has been shown that BSA conjugated to a phenolic compound, rutin, induced an increase in IgE receptor expression on B cells, a response that was abrogated by addition of anti-IL-4 monoclonal antibody³² indicating that the phenolic conjugate-mediated changes by altering IL-4 expression. Polyphenolic compounds have also been shown to enhance Th2 cytokines while inhibiting Th1 cytokines.^{15,32} Our cytokine data do not indicate that IL-4 or IL-13 production was elevated in the groups receiving walnut polyphenolic extract (Table 1). An increase in IL-5 production was observed (Table 1), but it was not significant over that seen with injection of OVA or OVA/AL, so it is unclear if the polyphenolics are increasing IL-5 and thereby potentiating IgE production. It is possible that the window to detect an increase in IL-4, IL-5, or IL-13 was missed, as it has been previously reported that cytokine production by splenocytes from transgenic mice sensitized to OVA peaks 1 week after the initial sensitization procedure.⁴⁴ Herein, cytokine production by splenocytes was evaluated nearly 4 weeks after initial sensitization of the mice. Additionally, cytokine measurements were taken from washed splenocytes obtained 9 days after polyphenolic exposure. Thus, our experimental conditions may not have allowed us to determine the impact of walnut polyphenolic administration on cytokine production.

Alternatively, the mechanism by which the Th2 antibody levels are increased in our mice may not be changes in cytokine expression but rather increased lymphocyte sensitivity to cytokines. The walnut polyphenolic extract may have mediated the increase in serum IgE and IgG₁ in an interleukin-independent manner through direct effects on antigen uptake and presentation, gene expression of IgE or IgE receptors, IgE degradation pathways, or by inhibiting interactions between IgE receptors and IgE molecules. Future studies in which these parameters are measured must be conducted before conclusions are drawn about the mechanisms by which walnut polyphenolic-enriched extract promotes the increase in IgE and IgG₁ antibody production.

In our experiments, mice were not evaluated for allergic reactions upon challenge with OVA. Mice were not challenged because it is unlikely there would be a measurable difference between the re-

sponses of the OVA/AL treated mice and the mice that received OVA/AL in the presence of polyphenolics due to the concentrations of IgE present in those mice.

Many routes of sensitization have been used in animal models of food allergy. Our choice of i.p. sensitization does not mimic the route of sensitization in human food allergy. However, others have successfully used the i.p. route of administration to determine the inherent allergenicity of food proteins⁴⁵ and have shown that i.p. administration is as effective and accurate as oral administration.^{46,47} They have shown a clear correlation between allergenicity and antigen-specific IgE production using i.p. sensitization.^{45,48} Additionally, an i.p. injection of a bolus of chlorogenic acid increased IgE as effectively as long-term oral exposure to chlorogenic acid.¹⁵ Herein, we wanted to determine the inherent Th2-skewing potential of a walnut polyphenolic-enriched extract. Thus, we began by testing i.p. administration of the substance in a previously established model of Th2 sensitization. Future research will be needed to determine if similar results are seen when antigen in the context of walnut polyphenolic-enriched extract is administered orally. However, on the basis of previously published research,^{15,46} we predict similar results will be seen by either route of sensitization.

We chose to use a model antigen, OVA, in lieu of using a walnut allergen for sensitization. In this way, we were able to separate the inherent sensitizing potential of walnut polyphenolic-enriched extract from the sensitizing potential of walnut protein. Additionally, use of an established model antigen enabled us to use fewer animals as no animal-intensive preliminary studies to determine the optimal quantity of walnut allergen or to determine which walnut allergen would best sensitize the mice were required. Now that we have established the ability of walnut polyphenolic-enriched extract to potentiate IgE responses, future work should include one or more of the established walnut allergens in the sensitization procedure.

It is possible that the concentration of polyphenolics injected i.p. may be higher than plasma concentrations achievable from the consumption of food with high polyphenolic content, but our interest lies in the gut microenvironment where 5 mM concentrations of polyphenolic compounds are possible.^{49–51}

We examined the effect of a food matrix component, polyphenolic compounds, on antibody response in BALB/c mice. Others have shown that components of the food matrix in peanuts and Brazil nuts are necessary for allergic sensitization.^{10,18} Although the specific component(s) of the food matrix that are required in allergic sensitization remain unidentified, these studies in combination with our own demonstrate that the food matrix can be important in potentiating immune responses in mice. Whether food matrix component(s) are required for allergic sensitization to foods in humans remains to be tested.

In conclusion, we have shown that i.p. coadministration of walnut polyphenolic-enriched extract with OVA in the presence of aluminum hydroxide gel can increase serum OVA-specific IgE and IgG₁ levels, without increasing serum OVA-specific IgG_{2a/2b} levels above that of the OVA/AL control. These effects are evidence for the potential of non-protein food matrix components to affect the allergenicity of proteins.

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Conflicts of interest

The authors declare no conflicts of interest.

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