

Functional analysis of cross-reactive immunoglobulin E antibodies: peanut-specific immunoglobulin E sensitizes basophils to tree nut allergens

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Summary

Background Peanut and tree nuts are a major cause of food-induced anaphylaxis with an appreciable mortality. Co-sensitization to peanuts and tree nuts is a common clinical observation and may be because of peanut-specific serum IgE antibodies that cross-react with tree nut allergens. It is, however, unclear whether these cross-reactive IgE antibodies are involved in effector-cell activation.

Objective To determine if cross-reactivity of peanut-specific IgE antibodies with tree nuts can cause effector cell activation using an *in vitro* basophil activation assay.

Methods Two peanut allergic subjects with positive specific IgE for peanut and tree nuts (as measured by CAP-FEIA) were studied. Basophil activation to peanut and tree nuts, as indicated by CD63 expression, was assessed by flow cytometry to confirm co-sensitization to peanut and tree nuts. Inhibition ELISA using sera from the subjects was performed to detect peanut-specific IgE antibodies that cross-reacted with tree nut proteins. To determine whether cross-reactive tree nut allergens can induce effector-cell activation, peanut-specific antibodies were affinity purified from the subject sera and used to resensitize non-peanut/tree nut allergic donor basophils stripped of surface IgE. Basophil activation was then measured following stimulation with peanut and tree nut extracts.

Results The two peanut allergic subjects in this study showed positive basophil activation to the peanut and tree nut extracts. Inhibition ELISA demonstrated that pre-incubation of the peanut allergic subject sera with almond, Brazil nut and hazelnut extracts inhibited IgE binding to peanut extract. IgE-stripped basophils from non-peanut/tree nut allergic subjects resensitized with affinity-purified peanut-specific antibodies from the peanut allergic subject sera became activated following stimulation with peanut, almond and Brazil nut extracts, demonstrating biological activity of cross-reactive IgE antibodies.

Conclusion Peanut-specific IgE antibodies that cross-react with tree nut allergens can cause effector-cell activation and may contribute to the manifestation of tree nut allergy in peanut allergic subjects.

Keywords allergen, basophil activation, cross-reactivity, IgE, peanut, tree nuts

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Introduction

Peanuts and tree nuts account for the majority of food-related anaphylaxis in children, adolescents and adults [1, 2]. Allergenic tree nuts include almond [3], Brazil nut [4], cashew [5], hazelnut [6], macadamia [7], walnut [8] and pine nuts [9]. Peanut allergy is more prevalent than tree nut allergy although clinical allergic reactions to multiple nuts are common. The reported prevalence of this type of food allergy within the general population varies widely. Sicherer et al. [10] reported that approximately 1.1% of the general population in the US is affected by peanut and/or tree nut allergy. Tariq et al. [11], in a cohort study involving 1218 children, reported

a similar sensitization rate of 1.2% to peanut and tree nuts, with 0.5% experiencing an allergic reaction upon subsequent ingestion of peanut and tree nuts. In specialist allergy clinics, the prevalence rate has been reported to be as high as 34–40% [12, 13]. It is apparent that the increasing consumption of peanuts has led to an increase in the prevalence of peanut allergy in the general population [14].

IgE cross-reactivity has been demonstrated between a number of tree nuts, including almond, hazelnut and walnut [15], macadamia and hazelnut [7] and walnut, hazelnut and Brazil nut [8]. We previously reported that peanut-specific IgE antibodies cross-react with allergens present in the tree nuts almond, Brazil nut and hazelnut when tested by inhibition ELISA [16]. Although these studies indicate that cross-reactive IgE antibodies may contribute to multiple sensitivities to peanut and various tree nuts, the effector-cell reactivity of these IgE antibodies has not been demonstrated.

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This is important, as the clinical relevance of some cross-reactive IgE antibodies has been questioned. In particular, there have been reports that IgE antibodies specific for cross-reactive carbohydrate determinants (CCDs) have poor biological activity and may contribute to false-positive results in *in vitro* tests for cross-reactivity [17–19]. However, the triggering of histamine release from basophils of tomato allergic subjects by glycoproteins purified from tomato extract suggests that CCD-specific IgE may be biologically active [20, 21]. Given the controversy surrounding the effector-cell activation by cross-reactive IgE antibodies, our study sought to determine whether peanut-specific IgE antibodies can activate basophils following exposure to cross-reactive tree nut allergens.

An *in vitro* assay has been optimized and used to assess effector-cell activation by peanut-specific IgE antibodies. In this assay, donor basophils were stripped of surface IgE and resensitized with affinity-purified peanut-specific antibodies. Resensitized cells were then stimulated with different tree nut extracts and basophil activation, as indicated by surface CD63 expression, was analysed. This stripped basophil activation assay was used to determine whether basophils resensitized with peanut-specific IgE antibodies, previously shown by inhibition ELISA to cross-react with tree nut allergens, could be activated upon exposure to tree nut extracts, demonstrating the biological significance of these cross-reactive antibodies.

Methods

Subjects

Whole blood and sera from two peanut allergic subjects recruited from the Alfred Hospital Asthma and Allergy Clinic, Melbourne, Australia were used in this study. These subjects had a clinical history of peanut allergy and positive peanut-specific IgE (CAP score of ≥ 2 (≥ 0.7 kU_A/L), Pharmacia CAP SystemTM, Pharmacia Diagnostics, Uppsala, Sweden). The study was approved by the Alfred Hospital Ethics Committee and informed consent was obtained before inclusion. The clinical characteristics and peanut/tree nut-specific IgE CAP scores for these subjects are given in Table 1. Both peanut allergic subjects presented with peanut-

induced anaphylaxis as the first evidence of a food allergy. This is reflected by their extremely high peanut-specific IgE levels. Peripheral blood was also obtained from two atopic, non-peanut/tree nut allergic subjects and their clinical characteristics are also outlined in Table 1.

Preparation of crude peanut and tree nut extracts

Crude peanut and tree nut extracts were prepared as described previously [16]. Briefly, commercially available almonds, Brazil nuts, cashews, hazelnuts and peanuts (either raw or roasted at 180 °C for 15 min, depending on the form usually ingested; Naytura, Sydney, Australia) were crushed and defatted using acetone and subsequently diethyl ether. The product was separated and dried by vacuum filtration and ground to powder using liquid nitrogen. Proteins were extracted by incubation in phosphate-buffered saline (PBS). For inhibition ELISA, CompleteTM protease inhibitor (Roche Diagnostics, Mannheim, Germany) cocktail was added to the PBS for extraction. The extracts were centrifuged to obtain a clear supernatant and the protein concentration was determined using the Bio-Rad Micro protein assay (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions.

Affinity purification of peanut-specific antibodies

Ninety-six-well polystyrene plates (Costar, Acton, MA, USA) were coated with roasted peanut extract (diluted to 1 µg/mL in 50 mM bicarbonate buffer; 50 µL/well) and incubated overnight at 4 °C. Plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 5% skim milk powder (SMP) in PBS-T (200 µL/well) for 1 h at 37 °C. Plates were again washed with PBS-T and incubated with subject sera (diluted 1/10 with 1% SMP in PBS-T; 50 µL/well) at 37 °C for 2 h. Plates were washed with PBS-T. Antibodies were eluted by adding 50 µL of 0.2 M glycine buffer (pH 2.6) containing 1% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) into each well followed by incubation at room temperature for 10 min, with shaking. The antibody solution was collected and neutralized to pH ~ 7.4 with 2 M NaOH. The specificity of the purified antibodies was assessed by ELISA as described below, using house dust mite (HDM;

Table 1. Clinical characteristics of peanut allergic and atopic, non-peanut allergic subjects

Subject no.	Age (years)	Sex	Atopy history	Skin prick test (mm)			Clinical history			CAP-FEIA score (kU _A /L)*				
				Mixed pollen	HDM	Cat	Known peanut/tree nut allergies	Other food allergies	Nature of reaction to peanut	Peanut	Almond	Brazil nut	Cashew	Hazelnut
A1	49	F	Atopic	8	5	3	Peanut, cashew, hazelnut	Peas, coconut, sesame seed	GIT upset, asthma, laryngeal oedema, generalized urticaria	5 (83.9)	ND	2 (1.20)	2 (0.88)	1 (0.25)
A2	18	F	Atopic	3	3	0	Peanut	Peas, fish	Laryngeal oedema, generalized urticaria, facial angioedema	6 (> 100)	2 (1.79)	2 (1.44)	1 (0.39)	3 (8.45)
NA1	28	F	Atopic	4	0	0	None	None	None	0	0	0	0	0
NA2	26	F	Atopic	11	10	14	None	None	None	0	0	0	0	0

*Specific IgE was determined by ELISA for the atopic, non-peanut/tree nut allergic subjects
ND, not done; HDM, house dust mite; F, female.

kindly provided by Allergy Therapeutics Ltd., Worthing, West Sussex, England), rye grass pollen (RGP; Greer Inc., Lenoir, NC, USA) and roasted peanut extracts. HDM and RGP extracts were prepared as described previously [22, 23].

Direct immunoglobulin E and inhibition immunoglobulin E enzyme-linked immunosorbent assay

These assays were performed as described by previously [16]. Ninety-six-well polystyrene plates (Costar) were coated with roasted peanut, HDM or RGP extracts, (diluted to a concentration of 1 µg/mL with 50 mM bicarbonate buffer, pH 9.6) and incubated overnight at 4 °C. Plates were washed with PBS-T and blocked as described above. For the inhibition ELISA, subject sera (previously diluted with 1% SMP in PBS-T for an OD 490 nm reading of ~1.0 for roasted peanut extract) were pre-incubated with peanut and tree nut extracts or, as a control, keyhole limpet haemocyanin (KLH; Sigma) in the presence of protease inhibitors at a final concentration of 0.2, 1, 5, 25 and 125 µg/mL at room temperature for 2 h. The inhibition mixtures (including sera with no inhibitor as positive controls), whole sera (diluted 1/10) or purified antibodies (neat) were dispensed into wells and incubated at 37 °C for a further 2 h. Plates were washed with PBS-T and incubated with rabbit polyclonal anti-human IgE antibody (1 : 1000; DAKO, Carpinteria, CA, USA) followed by horseradish peroxidase-labelled goat anti-rabbit IgG antibody (1 : 1000; Promega, Madison, WI, USA), both for 1 h at 37 °C, with PBS-T washes in between incubations. IgE binding was detected using *o*-phenylenediamine tablets (Sigma) dissolved in 0.05 M phosphate-citrate buffer (Sigma) and the reaction was stopped by adding 4 M hydrochloric acid. The absorbance (OD) in each well was measured at 490 nm. Non-specific binding of antibodies was eliminated by subtracting the absorbance in control wells containing no antigen from the absorbance in antigen-coated wells. The inhibition of IgE binding was expressed as percentage inhibition and calculated using the following formula:

$$\begin{aligned} \text{\% inhibition} \\ = 100 - \left(\frac{\text{OD}_{490} \text{ of serum with inhibitor}}{\text{OD}_{490} \text{ of serum without inhibitor}} \times 100 \right) \end{aligned}$$

Assays were performed in triplicate and mean values with standard deviation are shown.

Stripping and resensitization of peripheral blood basophils

This method was adapted from that described by Kleine Budde et al. [24]. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood of atopic, non-peanut allergic subjects by Ficoll-Paque centrifugation and the cell concentration was adjusted to 5×10^6 cells/mL. Surface IgE was removed by incubating cells in an equal volume of lactic acid buffer (13.4 mM lactic acid, 140 mM NaCl, 5 mM KCl, pH 3.9), on ice, for 5 min. The solution was neutralized by adding two volumes of HEPES buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.2 mM K₂HPO₄, 5.5 mM glucose, pH 7.4) containing 5% fetal calf serum (FCS; CSL Biosciences, Melbourne, Victoria, Australia) and the cells were pelleted by centrifugation at 286 g for 5 min. This stripping and neutralization procedure was

repeated once more and cells were washed in HEPES buffer containing 5% FCS. The cells were resensitized by incubation with affinity-purified peanut-specific antibodies at 37 °C for 1 h (~200 µL per 2.5×10^6 cells), washed once in HEPES buffer containing 5% FCS and resuspended in HEPES buffer containing 1 mM CaCl₂, IL-3 (2 ng/mL; R&D Systems, Minneapolis, MN, USA) and 5% FCS to give a final concentration of 5×10^6 cells/mL.

Activation of basophils

This method was adapted from Sanz et al. [25]. Aliquots of whole blood or resensitized cell suspension (100 µL; 5×10^5 cells per test) were incubated with 20 µL of stimulation buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 7 mM CaCl₂, 3.5 mM MgCl₂, 0.1% BSA, pH 7.4) containing IL-3 (2 ng/mL) and heparin (500 IU/mL; David Bull Laboratories, Melbourne, Victoria, Australia) and incubated at 37 °C for 10 min. Allergen challenge was performed with the addition of 100 µL of peanut and tree nut extracts or in some cases RGP negative control antigen (diluted with stimulation buffer containing heparin and IL-3 to obtain the desired concentration) to each aliquot of cells followed by incubation at 37 °C for 20 min. Cells were also stimulated with 100 µL rabbit anti-human IgE antibody (DAKO; diluted 1/1000 in stimulation buffer containing IL-3 and heparin) and *N*-formyl-Met-Leu-Phe peptides (fMLP) (Sigma; diluted 1/200 in stimulation buffer containing IL-3 and heparin) as positive controls. The activation of basophils was stopped by incubating cells on ice for 5 min.

Fluorescent staining of activated basophils

This was also conducted as described by Sanz et al. [25]. Cells were incubated with normal goat serum on ice for 10 min to block non-specific binding of antibodies, stained with phycoerythrin-conjugated mouse anti-human CD63 (Caltag Laboratories, Burlingame, CA, USA) and fluorescein isothiocyanate-conjugated goat anti-human IgE (Caltag Laboratories), and then washed with FACS wash buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 0.27 mM EDTA, pH 7.3). For experiments using whole blood, red cells were lysed at this stage by adding red cell lysis buffer (154 mM NH₄Cl, 10 mM KHCO₃, 0.81 mM EDTA). After staining and, if necessary red cell lysis, cells were washed and analysed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and Cell Quest software (Becton Dickinson). To permit exclusion of non-viable cells from analysis, 7-amino-actinomycin D (Sigma) was added. Approximately 200 000 events were collected per test sample to acquire sufficient numbers of basophils (500–1000) for analysis. The cut-off for CD63⁺ staining was based on the discrimination of staining between the negative control (no antigen control) and positive controls (fMLP and anti-IgE stimulation).

Results

Basophils from peanut allergic subjects are activated by peanut and tree nut allergens

The ability of the peanut and tree nut extracts to activate basophils from subjects A1 and A2 was assessed to confirm

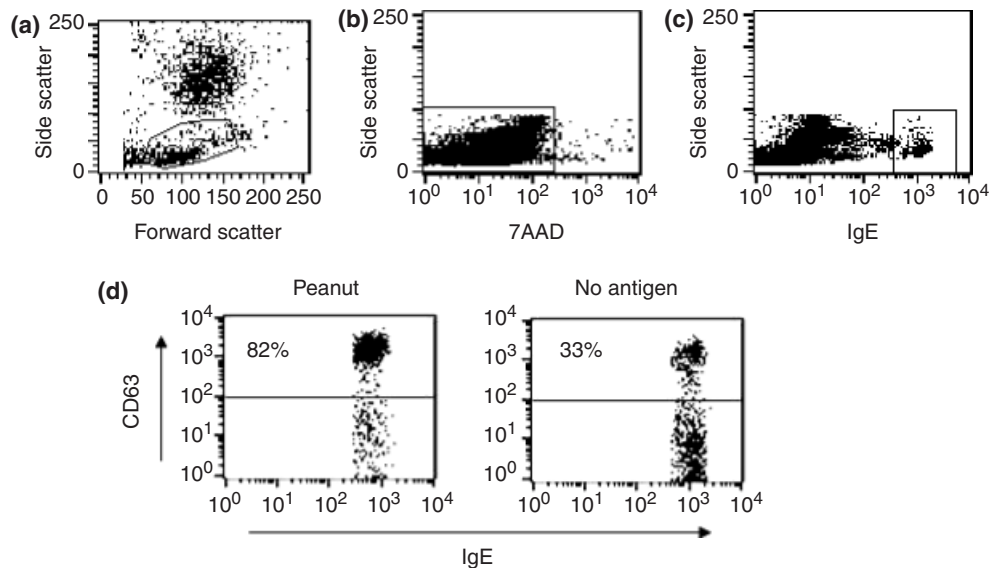


Fig. 1. Representative analysis of basophil activation following stimulation with peanut extract. Whole blood from a peanut allergic subject (A1) was incubated with roasted peanut extract (1 µg/mL) and the percentage of activated basophils was analysed via CD63 expression. (a) Cells were gated based on forward scatter and side scatter. (b) Live cells within this gate were selected via 7-amino-actinomycin D (7AAD) exclusion and (c) cells were analysed for high expression of IgE. (d) IgE^{hi} cells were analysed for CD63 expression and the percentage of activated basophils (upper quadrant) was calculated. A 'no antigen' control was included in the assay to determine baseline basophil activation.

Table 2. Percentage of activated basophils following stimulation with peanut and tree nut extracts

Subject no.	%CD63 ⁺ basophils					
	Roasted peanut*	Roasted Almond	Raw Brazil nut	Roasted Cashew	Roasted Hazelnut	No antigen†
A1	82	89	89	51	93	33
A2	57	53	45	23	55	26
NA1	0	0	0	0	0	0
NA2	1	0	0	0	1	1

*Positive control. †Negative control.

co-sensitization to peanut and tree nuts in these subjects. In this assay, whole blood was incubated with the peanut and tree nut extracts and the expression of CD63, a marker for activated basophils, was subsequently analysed by flow cytometry. A representative flow cytometric analysis is shown in Fig. 1. Stimulation of whole blood from peanut allergic subject A1 with roasted peanut extract resulted in a marked increase in CD63 expression among the IgE^{hi} cells (82%; Fig. 1d). In contrast, a much lower percentage of activated basophils (33%) was obtained in the absence of antigen stimulation (Fig. 1d).

Table 2 summarizes the percentage of activated basophils obtained for subjects A1 and A2 and the negative control subjects, NA1 and NA2. Positive basophil activation was obtained with roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut for subject A1, ranging from 51% to 93% which was above the 'no antigen' control cut-off. Similar results were obtained with subject A2 with 45–57% of basophils in whole blood becoming activated following stimulation with roasted peanut, roasted almond, raw Brazil nut and roasted hazelnut extracts. Negligible basophil activation was obtained with roasted cashew extract (23%) for this subject, which was similar to the 'no antigen' control (21%). Minimal basophil activation (0–1%) was also

observed with the non-peanut/tree nut allergic subjects following stimulation of whole blood with the peanut and tree nut extracts, demonstrating the specificity of this assay.

Peanut-specific immunoglobulin E antibodies cross-react with almond, Brazil nut and hazelnut allergens by enzyme-linked immunosorbent assay

Sera from two peanut allergic subjects (A1 and A2) were pre-incubated with different concentrations of peanut and tree nut extracts and inhibition of IgE binding to roasted peanut extract immobilized on ELISA plates was measured. As shown in Fig. 2, strong inhibition of IgE binding was obtained for both subjects when sera were pre-incubated with roasted peanut extract (positive control), demonstrating the specificity of this assay. Of the tree nuts tested, roasted almond extract showed the highest level of inhibition for both sera, followed by raw Brazil nut and roasted hazelnut extracts. Both almond and Brazil nut demonstrated greater than 50% inhibition of IgE binding to peanut, however, less than 50% inhibition was obtained with hazelnut even at the highest inhibitor concentration of 125 µg/mL. Minimal inhibition of IgE binding to roasted peanut extract was obtained with roasted cashew extract that was similar to the

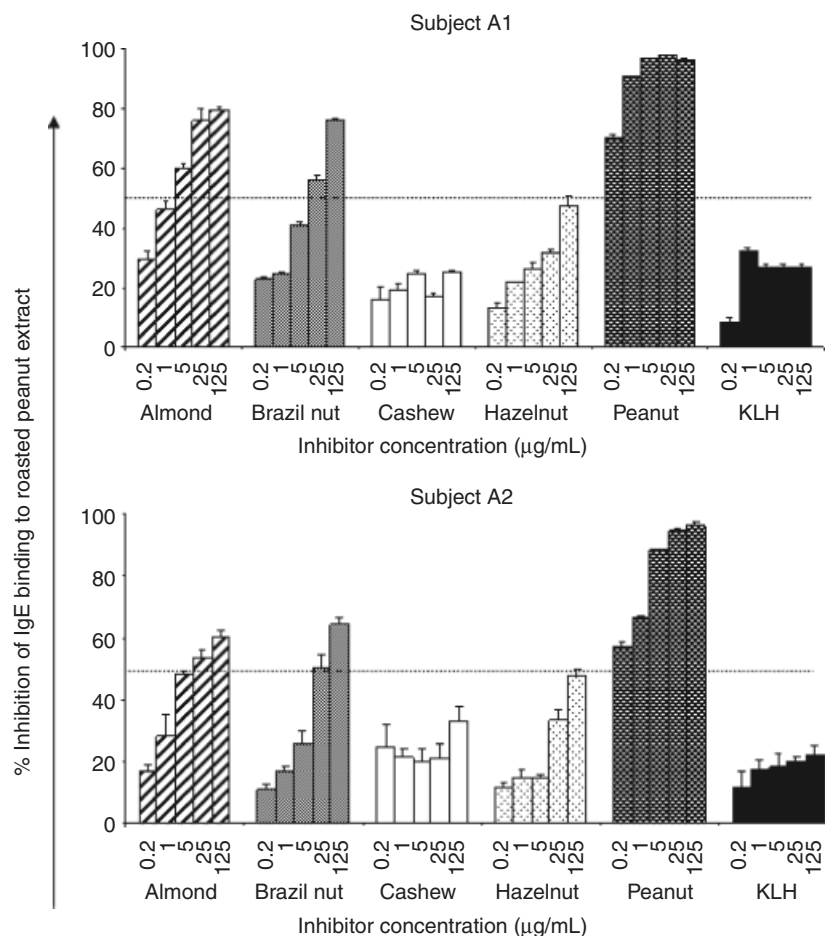


Fig. 2. Inhibition of IgE binding to peanut extract by tree nut extracts. Sera from two peanut/tree nut allergic subjects were pre-incubated with roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut, roasted peanut (positive control) and keyhole limpet haemocyanin (KLH) (negative control) extracts and inhibition of IgE binding to plate-immobilized roasted peanut extract was assessed. The mean values for triplicates are shown and the standard deviation is indicated by error bars. The dotted line indicates 50% inhibition of IgE binding to roasted peanut extract.

KLH negative control extract. The specificity of the observed inhibition was demonstrated by negligible inhibition of IgE binding to latex glove extract when serum from a latex, non-peanut/tree nut allergic subject was pre-incubated with the same concentrations of peanut and tree nut extracts (data not shown).

Measurement of the biological activity of cross-reactive peanut-specific immunoglobulin E antibodies

The biological activity of cross-reactive anti-peanut IgE antibodies was assessed using the stripped basophil activation test. Peanut-specific antibodies were purified following incubation of roasted peanut extract immobilized on ELISA plates with sera from subjects A1 and A2. The original serum from subjects A1 and A2 exhibited IgE reactivity to roasted peanut, HDM and RGP extracts but following affinity purification against peanut extract, there was minimal IgE reactivity to HDM and RGP extract while strong IgE reactivity to roasted peanut extract was maintained (data not shown).

Donor basophils from two HDM, non-peanut/tree nut allergic subjects (subjects NA2 and NA3) stripped of surface

IgE were resensitized with affinity-purified peanut-specific antibodies and subsequently stimulated with different concentrations of roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts. Figure 3 is a representative flow cytometric analysis for the stripped basophil activation assay. Incubation of IgE-stripped cells with peanut-specific antibodies did not result in increased IgE staining (Fig. 3a). However, a high number of CD63⁺ cells was detected when these resensitized cells were stimulated with roasted peanut extract, similar to the anti-IgE positive control, indicating a high level of basophil activation (Fig. 3b). This was not observed with the IgE-stripped cells, although basophil activation was detected with the anti-IgE stimulation, indicating the presence of IgE on the surface of basophils (Fig. 3b). Minimal basophil activation was also obtained with both the IgE-stripped and resensitized cells in the absence of antigen stimulation (Fig. 3b). Because of difficulties in distinguishing the IgE^{hi} cell population from other IgE-staining cells, basophil activation was expressed as the number of CD63⁺ cells.

To demonstrate that basophils from the non-peanut/tree nut allergic donors did not become activated to peanut and tree nut extracts before resensitization, donor cells stripped of

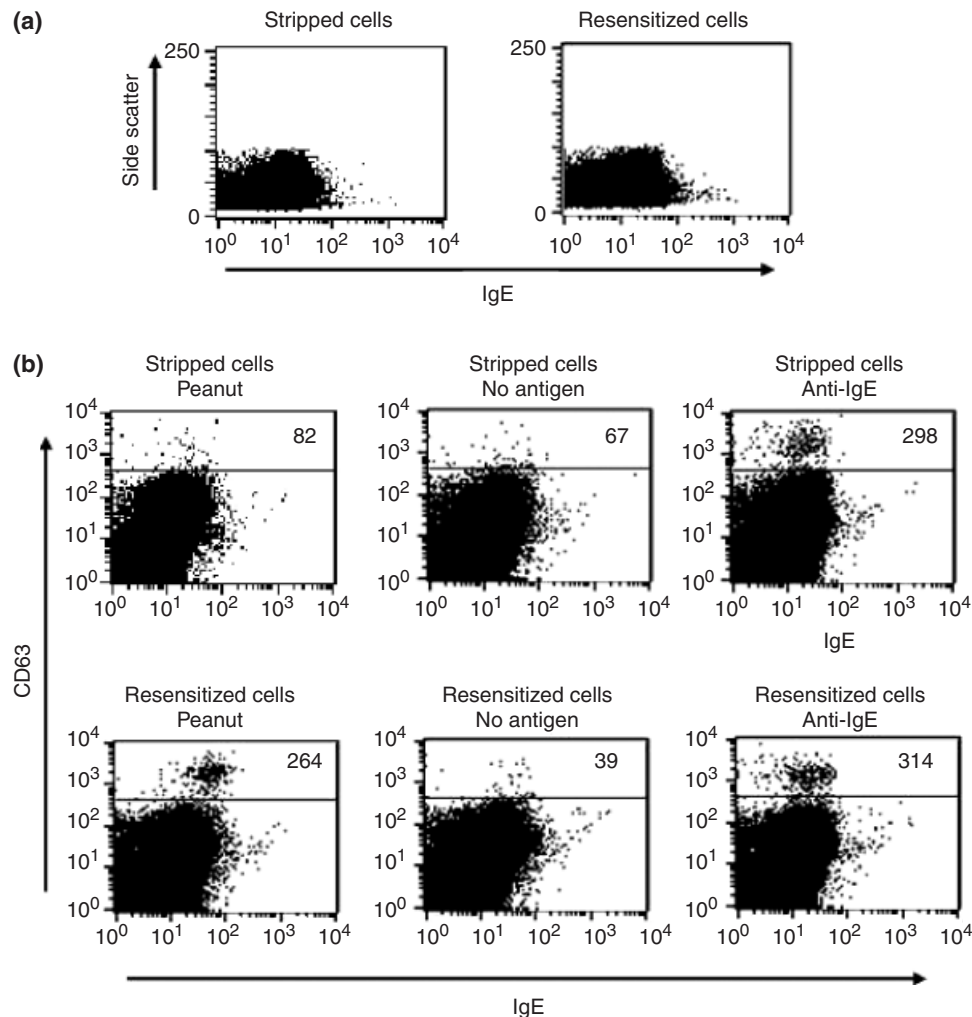


Fig. 3. Resensitization of IgE-stripped basophils with affinity-purified peanut-specific antibodies. Representative analysis of IgE-stripped cells following resensitization with purified peanut-specific antibodies (subject A2). After gating cells based on forward scatter, side scatter and 7-amino-actinomycin D (7AAD) exclusion, (a) IgE-stripped and resensitized cells were analysed for anti-IgE staining. (b) The number of CD63⁺ cells (upper quadrant) was then examined following stimulation with roasted peanut extract (10 µg/mL). A 'no antigen' control was included to measure background basophil activation. CD63⁺ cells were gated based on the discrimination of negative and positive control staining.

surface IgE were challenged with the peanut and tree nut extracts at the highest allergen concentration of 10 µg/mL. As illustrated in Figs 4a and b, high numbers of activated basophils were obtained upon stimulation with the positive controls, anti-IgE (199–298 CD63⁺ cells) and fMLP (386–981 CD63⁺ cells), demonstrating cell viability and function. Positive basophil activation to anti-IgE stimulation indicates that IgE antibodies are still present on the surface of the basophils even after the stripping procedure. However, minimal numbers of basophils (39–82 CD63⁺ cells) became activated following incubation of IgE-stripped cells with the peanut and tree nut extracts, similar to the 'no antigen' control.

Basophil activation was also observed when cells resensitized with purified peanut-specific antibodies from subject A1 and A2 sera were stimulated with anti-IgE and fMLP positive controls (Figs 4c and d), again indicating cell viability and function. However, in contrast to the IgE-stripped cells, stimulation of the resensitized cells with roasted peanut, roasted almond and raw Brazil nut extracts produced high

numbers of activated basophils, although a higher concentration of the tree nut extracts was required to obtain a similar level of basophil activation as the roasted peanut extract (Figs 4c and d). These data correlate with the inhibition ELISA results demonstrating cross-reactivity of peanut-specific IgE antibodies with almond and Brazil nut proteins. Minimal numbers of CD63⁺ cells were obtained following stimulation of the resensitized cells with RGP, similar to the 'no antigen' negative control (≤ 72 CD63⁺ cells), confirming the specificity of the purified peanut-specific antibodies from subject A1 and A2 sera.

Negligible basophil activation was obtained when cells resensitized with purified anti-peanut antibodies from subjects A1 and A2 were stimulated with different concentrations of roasted hazelnut extract, with levels similar to that obtained with the 'no antigen' and RGP negative controls (≤ 72 CD63⁺ cells; Figs 4c and d). The lower level of IgE cross-reactivity between peanut and hazelnut allergens in comparison with almond and Brazil nut allergens as described earlier may have contributed to this observation. A low number of

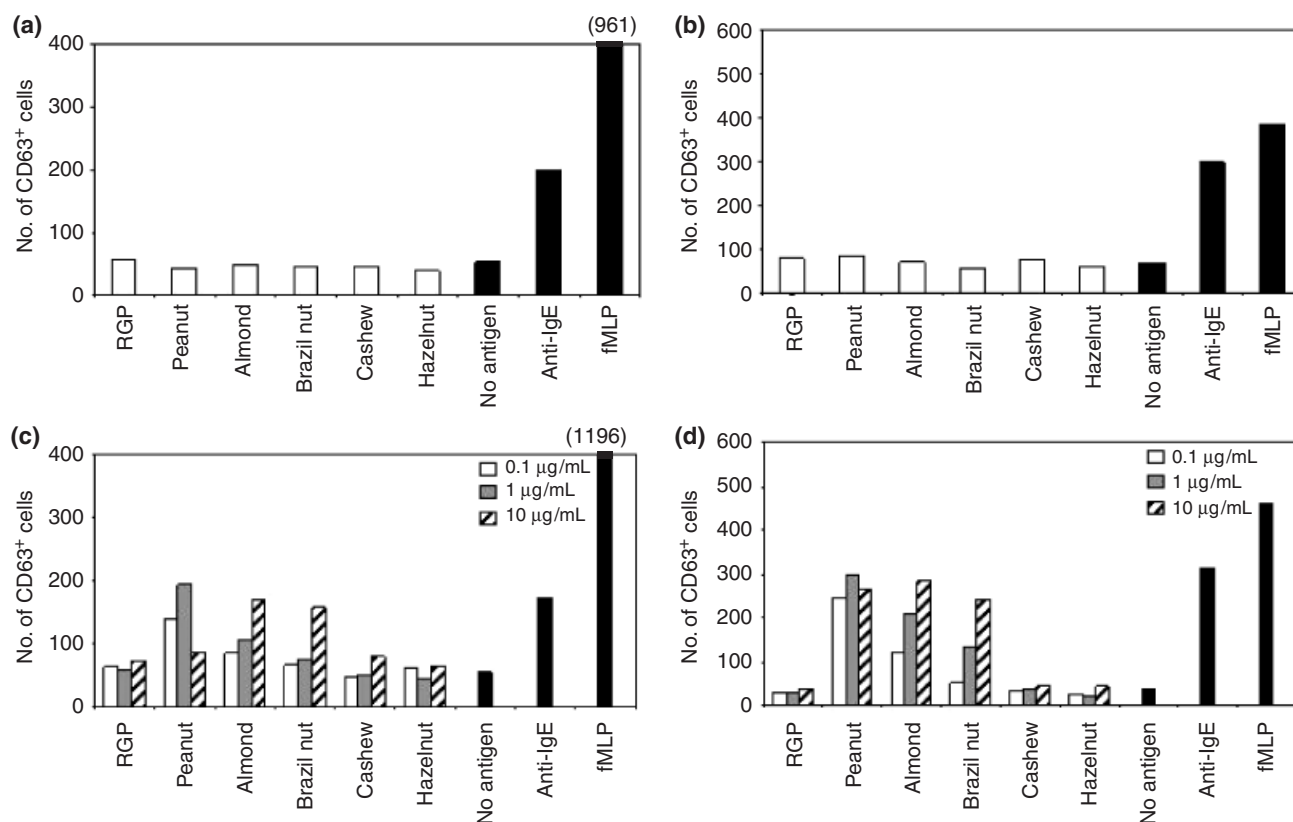


Fig. 4. Analysis of the biological activity of cross-reactive peanut-specific IgE antibodies from peanut allergic subjects. Partially purified basophils from atopic, non-peanut/tree nut allergic subjects (NA1 and NA2) were stripped of surface IgE and resensitized with peanut-specific antibodies purified from peanut allergic subject A1 and A2 sera. Cells were stimulated with roasted peanut, roasted almond, raw Brazil, roasted cashew and roasted hazelnut extracts and the number of cells expressing CD63 was analysed. Rye grass pollen (RGP) extract was included as a negative control and the positive controls were anti-IgE and *N*-formyl-Met-Leu-Phe peptides (fMLP) stimulation. (a) IgE-stripped cells from subject NA1 stimulated with allergen extracts (10 µg/mL). (b) IgE-stripped cells from subject NA2 stimulated with allergen extracts (10 µg/mL). (c) IgE-stripped cells from subject NA1 resensitized with peanut-specific antibodies from subject A1 serum stimulated with different concentrations of allergen extracts. (d) IgE stripped cells from subject NA2 resensitized with peanut-specific antibodies from subject A2 serum stimulated with different concentrations of allergen extracts.

activated basophils (34–80 CD63⁺ cells) was also obtained with roasted cashew extract even in the context of a positive clinical history and positive autologous basophil activation to this tree nut in subject A1. This supports the absence of IgE cross-reactivity between peanut and cashew and also serves as an additional control for the specificity of the stripped basophil activation assay.

Discussion

Co-sensitization to peanut and tree nuts is a common clinical observation among the peanut allergic population [12, 13] and the incidence of this type of food allergy is increasing [14]. Several studies have suggested that the multiple sensitivities to peanut and/or various tree nuts exhibited by some patients may be because of IgE antibodies that cross-react with peanut and tree nut allergens [7, 15, 16, 26–30]. It is, however, unclear if these cross-reactive IgE antibodies are involved in effector cell activation upon allergen exposure. Although inhibition ELISA can measure cross-reactivity using serum IgE antibodies, it does not characterize IgE antibodies present on effector cells such as basophils or their biological function. Some IgE-reactive allergen molecules are known to be weak

inducers of effector cell activation as a consequence of epitope orientation, preventing the cross-linking of surface IgE [31]. Therefore, cross-reactive tree nut allergens that bind peanut-specific IgE antibodies, as detected by inhibition ELISA, may not necessarily mediate cross-linking of effector-cell bound IgE antibodies. Consequently, an *in vitro* assay, the stripped basophil activation test, was established to determine effector-cell activation by peanut-specific IgE antibodies upon exposure to tree nut allergens.

In previous applications of a stripped basophil activation assay to investigate the biological activity of IgE antibodies and allergen preparations, histamine release from purified basophils was measured [20, 24, 32]. The use of flow cytometry to measure basophil activation eliminates the need to obtain purified basophils as these cells can be identified in a heterogeneous cell population by light scatter and surface marker staining [25]. The stripped basophil assay requires the availability of donor basophils and sufficient amounts of allergen-specific antibodies purified from subject sera for cross-reactivity studies. In our experience, the high affinity of some allergen-specific IgE antibodies can make purification by low pH elution difficult and only limited amounts of purified antibodies are obtained from some sera. In the two peanut allergic cases described here, a sufficient amount of

peanut-specific IgE antibodies was purified, allowing resensitization of basophils and a detectable level of activation upon allergen stimulation.

In this study, the level of cross-reactivity between peanut and tree nut allergens identified by inhibition ELISA did correlate with the degree of effector-cell activation in the stripped basophil activation assay. The number of activated basophils resensitized with peanut-specific antibodies was much higher with almond and Brazil nut compared with hazelnut. This correlated with the high level of IgE cross-reactivity observed with the inhibition ELISA where both almond and Brazil nut demonstrated greater than 50% inhibition of IgE binding to peanut. A high degree of IgE cross-reactivity is probably because of highly homologous allergens which contain multiple cross-reactive IgE-binding epitopes capable of eliciting the production of cross-reactive IgE antibodies. Multiple cross-reactive epitopes would inevitably result in increased epitope density, facilitating multivalent antigen-antibody interactions that are essential for IgE cross-linking and subsequent triggering of basophils. In contrast, low level cross-reactivity as observed with peanut and hazelnut where less than 50% inhibition of IgE binding was obtained, may be the result of low level sequence homology between the two allergens and fewer cross-reactive IgE-binding epitopes. As a result, such cross-reactive antigen-antibody interactions may be detected using IgE-binding assays but not cause efficient cross-linking of effector cell-bound IgE antibodies.

Effector-cell activation by cross-reactive IgE antibodies may also be affected by allergen abundance and the affinity of cross-reactive IgE antibodies for the cross-reactive allergen. These may be interrelated in that high allergen concentrations may be required to trigger basophils through low-affinity IgE antibody interactions. This may explain the inability of hazelnut extract to activate basophils sensitized with peanut-specific IgE antibodies even in the context of low-level cross-reactivity as measured by inhibition ELISA. Epitope orientation and density can also account for the differences in cross-reactivity as measured by IgE-binding assays and allergen-induced effector cell activation. This has been suggested as a possible reason for the discrepancy between IgE binding and effector cell activation as has been reported for a number of allergens [31].

Although this study does not address the identity of the cross-reacting allergens, possible candidates include a number of proteins that belong to the same family and are known to be allergenic in peanut and tree nuts. Ara h 2, a major peanut allergen, is a member of the conglutin family of proteins [33] that has been identified as allergenic in almonds [15]. The minor peanut allergen, Ara h 3, is classified as a legumin [34], also shown to contribute to the allergenicity of hazelnuts [6, 35]. Whether conglutins and legumins in peanut and tree nuts share similar IgE-binding epitopes is not known and requires further investigation. Carbohydrate moieties of allergens are known to contribute to IgE cross-reactivity between different allergen sources [36] although this appears unlikely for peanut and tree nuts. A study by van der Veen et al. [19] demonstrated that serum IgE from a number of grass pollen allergic subjects cross-reacted with peanut proteins even in the absence of clinical sensitivity. These cross-reactive IgE antibodies were found to be specific for carbohydrate

determinants and were poor inducers of histamine release [19]. In contrast, the majority of peanut allergic subjects had minimal IgE antibodies directed at the carbohydrate groups of peanut proteins [19].

We have demonstrated that *in vitro* cross-reactivity of peanut-specific IgE antibodies with almond and Brazil nut allergens can cause effector-cell activation. The *in vitro* cross-reactivity data may not correlate entirely with the clinical situation. Because of their high level of sensitivity to peanuts with known clinical co-sensitization to hazelnut and cashew (A1) and peas, coconut and sesame seed (A2), these patients vigorously avoid exposure to peanuts and tree nuts. Thus, although it is not apparent from the clinical history that these subjects are clinically sensitive to almond and Brazil nut, it is not ethically acceptable to challenge them because of the high potential for adverse reactions. Nevertheless, the specific IgE and basophil activation data clearly indicate that the subjects in this study are sensitized to almond and Brazil nut proteins and are likely to be susceptible to an allergic reaction upon exposure to these tree nuts. Both subjects suffered severe reactions to peanuts/tree nuts at an early age and were extremely careful not to ingest peanuts or tree nuts. It is therefore highly likely that the detection of specific IgE to tree nuts is because of allergen cross-reactivity. This study has demonstrated, using a stripped basophil activation assay, that cross-reactive peanut-specific IgE antibodies can cause effector-cell activation, indicating that avoidance of tree nuts is appropriate.

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