

ORIGINAL PAPER

Ses i 6, the sesame 11S globulin, can activate basophils and shows cross-reactivity with walnut *in vitro*

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Clinical and Experimental Allergy

Summary

Background Sesame allergy is increasingly being reported, and multi-sensitization to peanut and tree nuts has been described. The clinical relevance and cross-reactivity of many sesame proteins, such as Ses i 6, are unknown.

Objective The aims of this study were to perform a preliminary examination of the cross-reactivity of Ses i 6 *in vitro*, examine the ability of Ses i 6 to activate basophils in a modified basophil activation test (mBAT), and assess whether such an assay may help to distinguish between potentially relevant and irrelevant IgE reactivity towards 11S globulin proteins.

Methods Inhibition immunoblotting and chicken anti-rJug r 4 antibodies were used to determine the cross-reactivity of rSes i 6. Basophils from atopic donors were stripped of resident IgE before passive sensitization with food-allergic sera and challenged with protein extracts or recombinant protein. Basophil activation was measured using two activation markers, CD203c and CD63, via flow cytometry.

Results IgE immunoblotting showed cross-reactivity between rJug r 4 and rSes i 6 using sera from two human donors and chicken IgY. Additionally, rSes i 6 activated basophils passively sensitized with sesame-allergic sera. Cross-reactive serum from a sesame-allergic but walnut-tolerant donor was not able to activate basophils when challenged by walnut extract despite IgE reactivity to walnut determined by immunoblotting.

Conclusions The sesame 11S globulin shows partial immunological cross-reactivity with walnut, and although it is classified as a minor allergen, activated basophils sensitized with serum from seven out of eleven sesame-allergic donors. Additionally, the mBAT may help distinguish between clinically relevant and irrelevant *in vitro* IgE cross-reactivity of seed storage proteins in nuts and seeds and thus warrants use in further studies.

Keywords basophil activation test, cross-reactivity, food allergy, Jug r 4, seed storage protein, sesame, Ses i 6, 11S globulin, walnut

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Introduction

Sesame allergy has become a growing concern in the last decade with a natural history similar to peanut and tree nut allergies [1]. The estimated prevalence of sesame allergy varies geographically but sesame-containing foods are becoming ubiquitous. Sesame oil has been used in the pharmaceutical and cosmetic industries based upon presumed low antigenicity [2]. Unfortunately, the antigenicity of sesame may not be as low as once assumed, as increasing use in North America and Europe has been paralleled by an increase in reported sesame-induced

allergic reactions [3–7]. This should be a serious concern due to the age of onset, severity of the reactions, and persistence of the allergy [8–11]. Sesame can be used in a number of forms including whole or crushed seeds, oil, or meal [12] and there are a number of rodent studies and human clinical trials examining the nutraceutical benefits of sesame seeds, oil, or purified components. Such benefits may include favourable anti-oxidant properties, positive effects on lipid profiles, and modulation of blood pressure [12–18]. With increased exposure to sesame in the United States, it is not surprising that there are increasing reports of sesame allergy in the literature.

Seed storage proteins serve as a source of amino acids during germination and some may also function as pathogen inhibitors [19]. Two major storage proteins in sesame seeds are the 11S globulin (α -globulin) and 2S albumin (β -globulin) [20]. The 2S albumin (cloned as a precursor protein) has been shown to be the major sesame allergen based on the prevalence of IgE binding to an aqueous extract [12, 21]. Recently, oleosins were also shown to be important allergens from sesame oil bodies associated with severe clinical reactions [22]. The importance of other IgE-binding proteins is uncertain. Multiple polypeptides in sesame seed extract at varying molecular weights appear to bind IgE but neither their identity nor their relevance has been definitively determined [23]. One seed storage protein in particular, the 11S globulin, is potentially another significant allergen in sesame seed. This may be particularly true in those individuals with co-existent tree nut allergy whose IgE is reactive to a cross-reactive protein, such as Jug r 4, an 11S globulin protein in walnut.

Although the sesame 11S globulin, Ses i 6, has been classified as a minor allergen based on the prevalence of sensitization among sesame-allergic individuals, its clinical relevance is unknown [12]. This study attempts to better characterize the potential clinical relevance and cross-reactivity of Ses i 6 with walnut using both immunoblot inhibition and a modified basophil activation test (mBAT).

Methods

Recombinant proteins and whole extracts

The recombinant Jug r 4 protein was prepared as described previously [24], and a description of the sesame 11S globulin cDNA library construction and clonal expression system has been published previously [20].

All protein extracts were prepared as described previously [25]. An additional sesame extract was prepared using phosphate-buffered saline (PBS) (pH 7.4). As the rSes i 6 polypeptides generated in *Escherichia coli* without the benefit of eukaryotic post-translational processing [20] did not match the expected deduced location of the mature subunits in the sesame extract, native Ses i 6 was purified by ion-exchange chromatography [26–29] to illustrate the location of the mature polypeptides. Whole sesame seed extract was applied on separate 1 mL HiTrap Q columns (AKTA protein purification system Pharmacia, Uppsala, Sweden) previously equilibrated with loading buffer [20 mM 3-morpholinopropanesulphonic acid (MOPS) pH 7.2]. Ses i 6 was eluted using a linear sodium chloride gradient in loading buffer up to 1 M in 20 mL at a flow rate of 0.5 mL/min. Fractions were collected and analysed on SDS-PAGE. MALDI spectra sequencing revealed that the isolated polypeptides were sesame 11S globulin. Figure 1 shows the banding pattern of the sesame recombinant, purified 11S globulin, and whole extract on a reducing gel.

Immunoglobulin E cross-reactivity by immunoblotting

SDS-PAGE and IgE immunoblotting were performed as described previously [25]; however, proteins used for immunoblotting were separated on 10% acrylamide gels. Additionally, nitrocellulose strips were incubated with the SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Strips were exposed for 1 min on a Kodak BioMax Light X-ray film (Rochester, NY, USA). For inhibition immunoblotting, serum samples were pre-absorbed with recombinant proteins (10 μ g/mL) and extracts (200 μ g/mL) prepared in blocking solution. The concentrations of inhibiting protein used to pre-absorb sera were chosen based on the minimum concentration necessary to absorb out antibody reactivity to target proteins without non-specific inhibition of serum antibodies. Inhibition immunoblots were also carried out using polyclonal IgY (Aves Labs, Tigard, OR, USA) generated against the walnut fusion protein, rJug r 4, and sesame recombinant, rSes i 6 (Fig. 2). Chicken IgY antibodies were pre-absorbed with recombinant proteins and extracts as well as with an *E. coli* lysate solution (100 μ g/mL).

Human subjects

The study was approved by the institutional review board of the University of California at Davis. Blood samples were drawn after informed consent from 37 patients with histories of potentially life-threatening systemic reactions

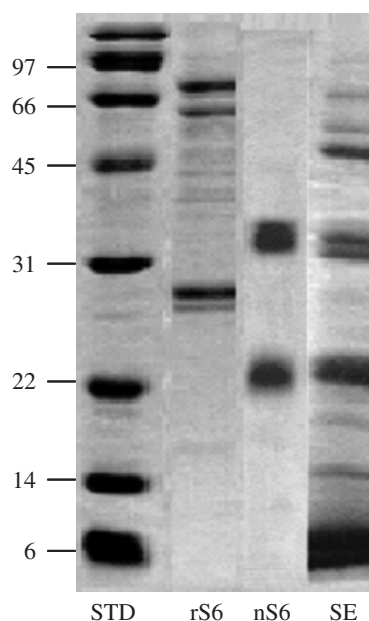


Fig. 1. Representative 14% SDS-PAGE reducing gel stained with Coomassie Brilliant Blue. STD, molecular weight standard; rS6, recombinant Ses i 6 fusion proteins; nS6, purified native Ses i 6; SE, whole sesame extract.

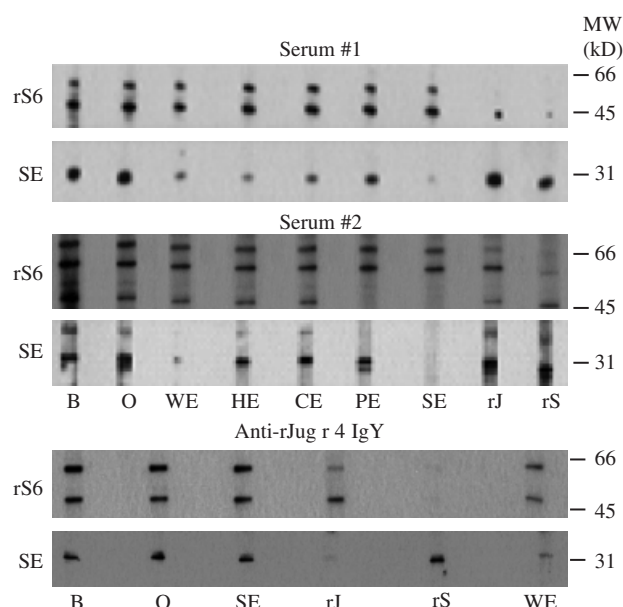


Fig. 2. Inhibition immunoblot of rSes i 6 (rS6) and whole sesame extract (SE) separated by SDS-PAGE using 10% acrylamide-reducing gels. Serum #1: from a patient with sesame, walnut, hazelnut, and cashew allergy. Serum #2: from a patient with sesame and peanut allergy. Sera and the chicken anti-rJug r 4 antibodies were pre-incubated with the following: B, blocking solution; O, ovalbumin; WE, walnut extract; HE, hazelnut extract; CE, cashew extract; PE, peanut extract; SE, sesame extract; rJ, rJug r 4 (walnut legumin); and rS, rSes i 6 (sesame legumin).

to peanut, walnut, and/or sesame involving bronchospasm, hypotension, or laryngooedema/throat swelling. Food challenges were not performed in these individuals due to the severity of reactions. Information on tolerance or allergy to other nuts and seeds, or whether they had never knowingly consumed other nuts or seeds, was also obtained. All serum samples were subjected to IgE immunoblotting to document the presence of IgE to sesame and walnut extracts, and to screen serum samples for IgE to the 11S globulin fusion proteins for each. Of the 37 patients, 19 reported reactions to sesame. Of the 19 reportedly reactive to sesame, 11 had IgE that bound to the recombinant 11S globulin from sesame, rSes i 6. The 11 with IgE specific to rSes i 6 were further tested using the mBAT, and seven were positive. Twenty-three of the 37 patients reported reactions to walnut, of which 14 reacted to the recombinant 11S globulin from walnut, rJug r 4. The 14 sera with IgE specific to rJug r 4 were tested using the mBAT and seven were positive. Six of the seven sera were donated from patients who did not report any reactions to sesame. Two serum samples, one from a patient with walnut and sesame allergy, and one from a patient with sesame allergy who tolerates walnut but has IgE directed to the 11S globulin protein from each, were studied in further detail. The sera from peanut-allergic patients were used as additional positive controls in determining the cut-off point for a positive mBAT. The

sera were frozen at -70°C until use. Atopic volunteers with allergic rhinitis but no food allergies donated blood samples for the mBAT.

Modified basophil activation test

Basophils were enriched from whole blood collected from non-food-allergic, but atopic, human donors in a single step using a Percoll gradient as has been described previously [30]. Pelleted basophils were re-suspended in an ice-cold pH 3.9 lactic acid buffer containing 13.4 mM lactic acid, 140 mM NaCl and 5 mM KCl [31] for 1 min before washing in PBS. The cells were re-suspended in a 1 mL solution containing 20% sera from food-allergic donors, 4 mM EDTA, 10 $\mu\text{g/mL}$ heparin, and placed in a heated water bath for 90 min at 37°C [32]. Before allergen challenge, basophil solutions were washed with a 20 mM HEPES buffer, pH 7.4, [32] and incubated in 150 μL of the HEPES buffer containing 1 mM CaCl_2 for 30 min at 37°C . Basophils were challenged for 15 min with 5 $\mu\text{g/mL}$ of recombinant allergen (serial dilutions from 0.04 to 25 $\mu\text{g/mL}$ were initially screened and 5 $\mu\text{g/mL}$ produced the greatest reactivity without non-specific activation), pelleted, and re-suspended in staining buffer. Mixed in with the staining buffer was Fc block (Milenyi Biotec, Auburn CA, USA), 7-AAD (BD Pharmingen, Franklin Lakes, NJ, USA), phycoerythrin (PE)-conjugated anti-human CD203c (Immunotech, Marseille, France), and fluorescein isothiocyanate (FITC)-conjugated CD63 (Serotec, Raleigh, NC, USA) using individually titred concentrations. Samples were analysed on a Becton Dickinson (Franklin Lakes, NJ, USA) FACScan using FlowJo (Tree Star Inc, Ashland, OR, USA) software. Each mBAT included a positive control, formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma, St Louis, MO, USA), a negative control, PBS, and an irrelevant allergen. Tree nut, peanut, and sesame extracts as well as recombinant legumin proteins were used as irrelevant allergens for serum from patients with reported tolerance to the food and whose sera were devoid of any IgE reactivity to the corresponding protein extract. In samples from multi-reactive patients with IgE reactivity to multiple nut and seed extracts, ovalbumin was used as the irrelevant allergen.

Data analysis

Flow cytometry analysis was performed on a minimum of 250 000 events per sample and cells were gated for 7-AAD $^{-}$ CD203c $^{+}$ to identify viable basophils. To determine basophil reactivity, the percentage of 7-AAD $^{-}$ CD203c $^{+}$ CD63 $^{+}$ cells compared with total 7-AAD $^{-}$ CD203c $^{+}$ cells was converted into an activation index (AI). The AI is presented as a ratio representing the percent increase of 7-AAD $^{-}$ CD203c $^{+}$ CD63 $^{+}$ basophils in challenged samples compared with unchallenged

basophils. An AI ≥ 0.25 (i.e. a 25% increase in 7-AAD⁻ CD203c⁺ CD63⁺ basophils from control) was used as a cut-off value in our laboratory based on preliminary experiments with positive and negative controls to optimize the assay. Wilcoxon rank sum tests were carried out using SigmaStat 3.0 software.

Results

The sesame 11S globulin, Ses i 6

Figure 1 shows the banding pattern of the sesame recombinant, the purified 11S globulin, and the whole extract on a reducing gel. The 11S globulins in seeds are generally encoded by a family of genes producing precursor proteins that are cleaved post-translationally. Although a single protein (and usually isoforms), the subunits separate on denaturing, reducing gels such as those we used. The bands in nS6 were both sequenced using LC MS/MS and were identified as sesame 11S globulins (top band; gi|5381325, bottom band; gi|81238592). We now know that the top band is the acidic subunit from up to four identified isoforms of Ses i 6, and the bottom band is the basic subunit from these four isoforms [33]. Although the whole sesame extract does not share corresponding bands based on molecular weights with the recombinant fusion protein precursor (before post-translational processing), it does share bands with the purified protein, lane nS6, as expected.

Cross-reactivity between rSes i 6 and rJug r 4

In Fig. 2, the results of the IgE inhibition immunoblots of two sera are compared, showing only the region of the blot with cross-reactivity. It is important to point out that the single band observed in the SE lanes likely represents the acidic subunit of Ses i 6. Not all patients have IgE that bind both the acidic and basic subunits of legumin proteins. In addition, cross-reactive IgE from patients reactive to legumin proteins can sometimes be limited to one of either the acidic or basic subunits. Only the acidic subunit is shown here because only the acidic subunit displayed cross-reactivity in these two patients. Serum #1 is from a patient with a self-reported history of life-threatening reactions to walnut and hazelnut, and has also reported reactions to sesame and cashew. Serum #1 has IgE reactivity to peanut (1.06 kIU/L by ImmunoCAP) but the patient is clinically tolerant of peanuts. Reactivity to rSes i 6 was marginally reduced when IgE was pre-absorbed with both the walnut and sesame extracts but was nearly eliminated when pre-absorbed with recombinant 11S proteins from walnut and sesame. Walnut and hazelnut extracts reduced IgE reactivity (more so than peanut and cashew extracts) to the only polypeptide in the sesame extract to which this patient's serum IgE bound. Interestingly, rSes i 6 was unable to inhibit the IgE binding

to the single band in the sesame extract, suggesting that differences in IgE epitopes exist between the recombinant sesame 11S globulin and the native.

Serum #2 was donated by an individual who is clinically tolerant of walnuts but does experience severe reactions upon consumption of sesame seed. This patient has a history of anaphylactic responses to peanut (the initial allergy reported, ImmunoCAP 49.5 kIU/L), but has never eaten hazelnut or cashew. As shown in Fig. 2, the walnut extract was able to significantly inhibit IgE reactivity towards the three bands in sesame extract with which serum #2 is reactive. The peanut, hazelnut, and cashew extracts, however, showed only partial inhibition. The peanut extract was able to inhibit IgE binding to only the higher molecular weight polypeptide in the sesame extract. There was also removal of a small band when pre-absorbed with hazelnut and cashew that was not affected by pre-absorption with peanut extract or controls. There was little or no inhibition of IgE reactivity to the sesame extract when pre-absorbed with the recombinant proteins at the concentration used.

Serum #2's IgE reactivity towards rSes i 6, however, produced a very different profile compared with serum #1. Only pre-absorption of serum #2 with rSes i 6 was able to significantly decrease IgE binding to the recombinant protein, albeit incompletely. The results of the rSes i 6 blots show reduced binding to one of the two high-molecular-weight bands when pre-absorbed with rJug r 4. rSes i 6 inhibition was not seen after pre-incubation with walnut extract. The peanut and sesame extracts, conversely, eliminated binding to the lowest molecular weight band.

Chicken antibodies raised against rJug r 4 provided strong evidence to support the cross-reactivity between the 11S globulins of walnut and sesame. The anti-rJug r 4 IgY antibodies were able to bind the multiple truncated fusion protein products of rSes i 6 (Fig. 3), but with apparent differences in titre recognizing the various truncated fusion proteins as compared with the anti-rSes i 6 IgY antibodies. Interestingly, in Fig. 2, pre-incubation of the anti-rJug r 4 IgY antibodies with rJug r 4 decreased IgE binding only to the two higher molecular weight polypeptides of rSes i 6 (only these two bands showed cross-reactivity and so are the only bands shown in Fig. 2). This implies that rJug r 4 in solution did not contain enough accessible relevant epitopes to absorb out IgY activity to the sesame legumin when denatured, reduced, and bound to nitrocellulose. Pre-incubation with walnut extract also appeared to mildly reduce reactivity to both bands. This high-molecular-weight fraction in rSes i 6 is likely the band appearing around 55 kDa in Fig. 3 in which proteins were separated with a 14% acrylamide gel. The reactivity of chicken antibodies was also reduced towards the band at about 31 kDa in the whole sesame extract (to which the two patient serum samples were also reactive), after incubation with rJug r 4 and walnut extract.

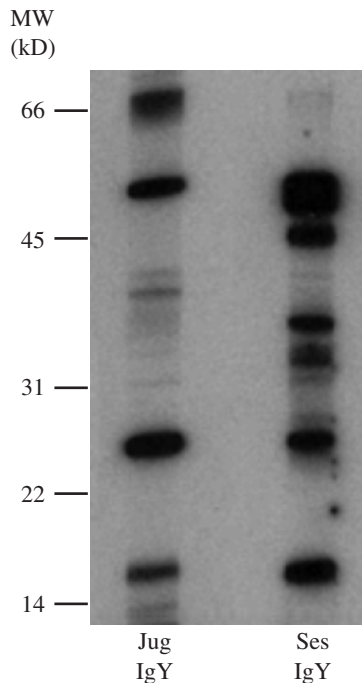


Fig. 3. Western blot of rSes i 6 separated by SDS-PAGE under reducing conditions on a 14% acrylamide gel using chicken antibodies. This immunoblot compares the reactivity of chicken antibodies raised against rJug r 4 (Jug IgY) and rSes i 6 (Ses IgY) towards rSes i 6.

Modified basophil activation test

Using serum samples from our sera bank, we were able to develop an mBAT using sera from donors with documented medical histories of severe reactions to peanuts, tree nuts, and sesame seeds. To compare the relative reactivity of passively sensitized basophils using sera from multiple food-allergic donors, a single allergen concentration was chosen for mBAT experiments. This concentration was determined using serial dilutions of allergen (Fig. 4). The concentration that produced the greatest reactivity without non-specific activation was chosen and used for all mBAT assays using the same allergens (5 µg/mL).

The results of experiments with 37 sera are listed in Table 1. Basophils donors were atopic, non-food-allergic individuals. Each basophil donor was challenged with each of the allergens (peanut, sesame, and walnut) as were the basophils sensitized with food-allergic serum (37 sera donated from patients with self-reported reactions to peanut, sesame, and/or walnut). A comparison of the mBAT results from these two groups is shown in Table 1 (control donors vs. sera donors). For every allergen used in the mBAT, the percent increase of activated basophils (reported as the activation index) was significantly higher in samples containing basophils sensitized with IgE from food allergic donors than from control donors (Table 1). In addition, there were no significant differences between mBAT results from control donors and food allergic

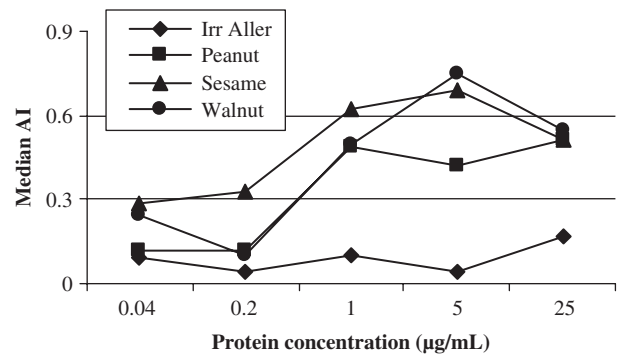


Fig. 4. Dose-response curves for peanut, sesame, and walnut extracts. Serum IgE from 37 patients with a self-reported history of food allergy to walnut and/or sesame was used to passively sensitize human basophils and challenged with serial dilutions of allergen (0.04–25 µg/mL). The activation index (AI) is plotted on the y-axis and represents a ratio of the percent increase of 7-AAD⁻ CD203c⁺ CD63⁺ basophils from control after challenge. The same protein solutions were used to challenge sera from patients who reported tolerance to either walnut or sesame and are referred to as irrelevant allergens (Irr Aller). A 5 µg/mL was chosen for calculation of sensitivity and specificity data.

donors challenged with irrelevant proteins. Irrelevant proteins included those proteins from food to which the food allergic donors have reported clinical tolerance (either peanut, sesame, walnut, or ovalbumin). The mBAT effectively distinguished between food allergic and control donor serum samples for all three allergens tested (peanut, sesame, and walnut) and the allergens did not appear to non-specifically activate donor basophils nor did donor serum activate basophils during passive sensitization.

Table 2 represents the data used to help determine the appropriate cut-off for a positive activation index, based upon results from 37 serum donors with clinical histories positive for life-threatening allergic reactions to tree nuts, peanut, and/or sesame and six atopic, non-food allergic basophil donors. The data were divided between those samples challenged with the non-specific activator, fMLP, and those challenged with protein extracts expected to give a positive result based on the positive clinical history of the serum donor (walnut, peanut, and/or sesame). For the purposes of finding an optimal AI cut-off, these samples give the 'true positive' rate, or the 'sensitivity' for this set of data. The challenges with irrelevant allergens, expected to be negative, likewise generate the 'specificity' for this set of data, i.e., the true negatives, based on the clinical history. It was calculated from basophils challenged with the same protein extracts but using serum samples from donors known to be tolerant of one or more of the proteins. Although the mBAT could not generally achieve the high percent of activated basophils when challenging with allergens as with fMLP, using an AI of 0.25, i.e. a 25% increase in activated basophils over baseline, maintained reasonable sensitivity without losing

Table 1. Results of mBAT*

Challenge	Control donors [†] (n = 6)	Sera donors [‡] (n = 37)			P-value [§]
fMLP	0.91 (0.20–1.90)		1.01 (0.19–8.88)		
Food allergen		Peanut allergic n = 19	Sesame allergic n = 19	Walnut allergic n = 23	
Peanut	0.06 (–0.18–0.24)	0.70 (–0.12–5.89)	0.12 (0.06–0.25)	0.08 (–0.18–0.39)	0.02 , 0.93, 0.96
Sesame	0.04 (–0.18–0.45)	0.04 (–0.12–0.40)	0.74 (–0.01–3.43)	0.12 (–0.23–0.35)	0.818, 0.007 , 1.00
Walnut	0.08 (0.03–0.30)	0.09 (–0.25–0.31)	0.15 (0.12–0.31)	0.65 (–0.03–5.24)	0.836, 0.114, 0.013

*Data are expressed as the median activation index (AI) using duplicate measurements with the data range in parenthesis. The AI is presented as a ratio of the percent increase of control samples. An AI ≥ 0.25 (i.e. a 25% increase in 7-AAD[–] CD203c⁺ CD63⁺ basophils from control) is considered positive for basophil activation.

[†]Atopic, non-food-allergic donors.

[‡]Food-allergic donors.

[§]Statistical analysis of control donors versus sera donors for peanut, sesame, and walnut using the Wilcoxon rank sum test. A P-value < 0.05 and the corresponding median AI are in bold type.

Table 2. Determination of the optimal AI cut-off point for the data set*

Activation index if set at	Sensitivity (%) fMLP	Sensitivity (%)			Specificity (%)		
		Pea	Ses	Wal	Pea	Ses	Wal
0.25	89	71	81	81	78	82	83
0.10	94	79	88	89	44	45	57

*Sensitivity data was calculated for basophils activated non-specifically using fMLP and using allergen extracts: Pea, peanut; Ses, sesame; Wal, walnut. Specificity data represents basophils challenged with irrelevant proteins as controls. Previous studies using more traditional BATs report cut-off points in similar ranges [34–38].

fMLP, formyl-methionyl-leucyl-phenylalanine; BAT, basophil activation test.

specificity (Table 2). Increasing the sensitivity of the assay by using a lower decision point (0.10) resulted in a 30–40% loss of specificity and therefore 0.25 was chosen as the decision point for activation. It is important to point out that the data were calculated based on self-reported histories of patients and may differ from actual clinical reactivity. Therefore, the calculations here are a conservative estimate applicable only to these allergens at the concentration of allergen used for challenge.

Basophil reactivity of rSes i 6

Initially, 19 sera from patients with clinical histories of sesame allergy were screened for reactivity by immunoblotting to the recombinant protein, rSes i 6, and 11 (58%) appeared to have varying degrees of IgE reactivity to rSes i 6. Of the 11 reactive-sera, four were also known to have IgE reactivity to the walnut fusion protein, rJug r 4. Two sera were chosen from these four who were reactive towards both sesame and walnut 11S globulins based on availability of an adequate amount of sera; one serum was reactive towards rJug r 4 and had a severe walnut allergy by self-report (serum #1) and one serum was from a patient clinically tolerant of walnuts, but that showed IgE binding to rJug r 4 (serum #2).

A comparison of the reactivity of the extracts and 11S globulin fusion proteins of sesame and walnut is listed in Fig. 5. Serum #1 is from a patient who is reportedly

clinically reactive to both walnut and sesame seed, which is supported by the positive results of the mBAT to both recombinants. Serum #2, possessing cross-reactive IgE, is from a patient who is clinically tolerant of walnuts despite IgE reactivity, and when basophils sensitized with serum #2 were challenged with walnut extract or rJug r 4 at the same concentration of 5 µg/mL, there was no basophil reactivity, supporting the clinical observation that this patient has clinically irrelevant cross-reactive IgE to walnut 11S globulin. In addition to serum samples #1 and #2, nine additional sera (making a total of 11) from donors with IgE specific to rSes i 6 who reported reactions to sesame were used to confirm the potential clinical reactivity of rSes i 6, and indeed, rSes i 6 activated passively sensitized basophils from five of these sera (Fig. 5 'Sesame Sera'). Basophils sensitized with sera from 14 of the 23 clinically walnut-allergic patients known to have IgE reactivity towards rJug r 4 were also tested using the mBAT. Of the seven sera samples that were mBAT-positive when challenged with walnut rJug r 4, 6 came from donors without clinical sesame allergy and were not able to activate passively sensitized basophils when challenged with sesame proteins (Fig. 5 'walnut sera').

Discussion

The data from inhibition immunoblotting suggest that there are cross-reactive epitopes between the 11S

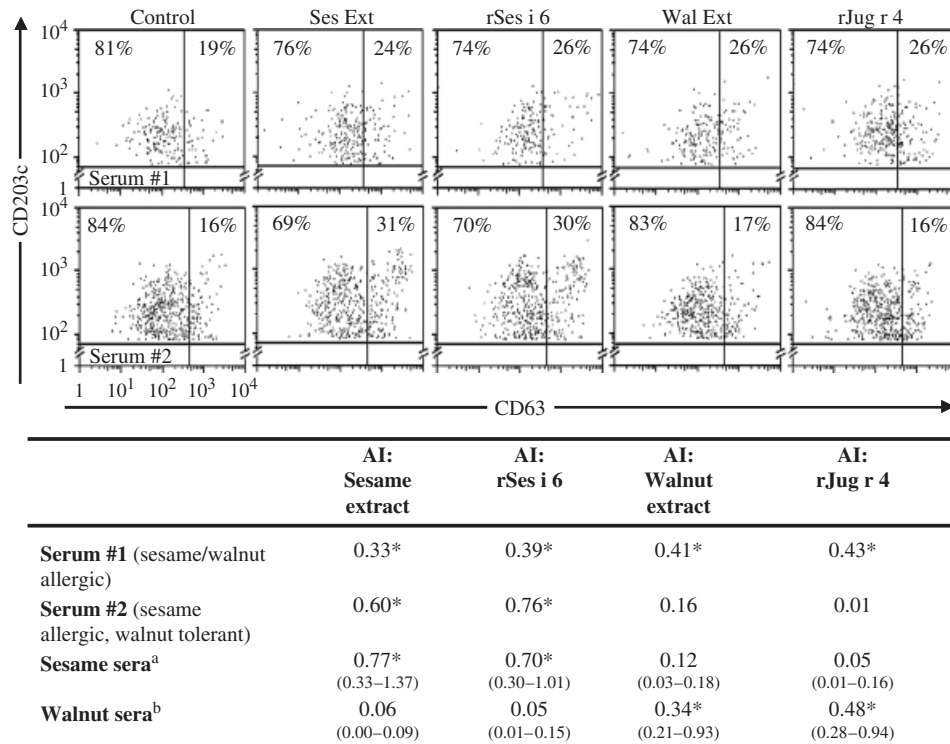


Fig. 5. mBAT results based on clinical history of food allergy to sesame or walnut in patients with IgE to 11S globulins. The dot plots represent the results of a representative mBAT using serum samples #1 and #2 with the control on the far left. *Represents a positive reaction (AI > 0.25). The data for Sera #1 and #2 are expressed as the median AI of duplicate measurements repeated two times. ^aMedian AI of 7 sera from sesame-allergic donors tolerant to walnut; ^bMedian AI of 6 sera from walnut-allergic donors tolerant to sesame. Data range is in parentheses.

globulins of sesame and walnut. Serum samples #1 and #2 were donated by sesame-allergic individuals and were reactive to rSes i 6 in the mBAT. However, both sera donors also showed IgE to rJug r 4, and yet only serum #1 was from a clinically walnut-allergic subject; thus, IgE to walnut in serum #2 was clinically irrelevant. The mBAT supported this finding as neither rJug r 4 nor walnut extract was able to activate basophils when this serum was used, even though walnut extract was able to almost completely inhibit IgE binding against sesame extract allergens on inhibition immunoblotting (Fig. 2, lane WE). Cross-reactivity was also supported by showing that chicken antibodies raised against rJug r 4 were able to bind rSes i 6 (after appropriate pre-absorption with *E. coli* lysate) and bound the predicted acidic subunit of the sesame legumin polypeptides in whole sesame extract (Fig. 2).

The 11S globulin represented by Jug r 4 appeared to have cross-reactive epitopes with only the predicted acidic subunit of the sesame legumin in a reduced sesame extract (as seen in Fig. 2), but chicken IgY raised against rJug r 4 bound the same truncated fusion protein products as chicken anti-rSes i 6, but with apparently different avidity or epitope titres, yielding a darker band for a few of the polypeptides and lighter bands for others. This

reactivity was completely eliminated by pre-incubation of the anti-rJug r 4 IgY with rSes i 6 (Fig. 2, lane rS). The same reaction was not observed with sesame extract, possibly the result of conformational differences between the whole extract and recombinant proteins. As serum #2 is from a patient who is tolerant to walnuts, the epitopes available for IgE binding on this polypeptide may be cross-reactive but may not be clinically relevant.

The cross-reactivity shown here between sesame and tree nut likely occurs in peanut as well. Peanut extract was able to reduce IgE reactivity from serum #2 towards one polypeptide of rSes i 6, and our lab is currently accumulating more data to show that these allergens are cross-reactive. This is not the first report to suggest cross-reactivity between sesame and peanut as Beyer et al. [23] showed sequence homology between IgE-binding epitopes in Ses i 3 and Ara h 1. It is important to note that 60% of the sesame-allergic children in France are multi-sensitized (usually to tree nuts and peanuts), but the clinical relevance of such positive skin tests has not been assessed [39]. Multi-sensitization can result from either independent sensitizations or IgE that is present to cross-reactive epitopes. Cross-reactive epitopes, which do not necessarily translate into clinical reactivity, could be responsible for a large proportion of sesame-allergic

individuals sensitized to seed storage proteins from other seeds [12, 20, 23, 40]. For example, the 2S albumins of peanut, walnut, Brazil nut, and soybean are all known food allergens. The 2S albumin from sesame shares approximately 35–40% homology with the 2S albumin precursor from Brazil nut [23, 41, 42], 45% with the precursor from castor bean [41, 42], 38% with the walnut allergen [23], and 34% with the peanut allergen Ara h 2 [23]. Such percentages may appear low, but when IgE-binding epitopes were identified by Beyer et al. [23] on the sesame vicilin (Ses i 3) (which, overall, has only 36% homology to the peanut allergen Ara h 1 and 41% homology to the walnut allergen Jug r 2), a surprising 80% homology with the corresponding area on Ara h 1 was found.

The results of this study provide preliminary evidence that modifications to traditional BAT could be used to help study cross-reactive food allergens. In combination with current assays (inhibition immunoblotting, ImmunoCAP-FEIA, skin prick testing, etc.), the mBAT may be a useful supplement for predicting clinical reactivity, but would require titration of each allergen. The cross-reactivity of allergens in tree nuts, peanuts, and sesame seed affects the accuracy of current *in vitro* methods [43–45] and besides oral food challenges, non-invasive strategies of allergy diagnosis have not yet achieved the specificity necessary to distinguish between clinically relevant and irrelevant IgE cross-reactivity. The mBAT using human basophil donors and passive sensitization may be a simpler, faster, and more biologically relevant assay compared with traditional BATs. Passive sensitization is a promising tool for analysing the reactivity of sera because the antibodies from sera are more stable and can be frozen, as opposed to working directly with patient basophils.

Rodent cell lines may not have the FcεRI receptor expression nor the reactivity of natural basophils, especially those from atopic individuals [46]. The mBAT also allowed pre-screening of donor basophils for non-releasers and avoided cytokine treatments that may interfere with activation markers [47, 48]. The assay is relatively quick, requiring 4–5 h from blood collection and even from a small blood sample several allergens per donor sera can be tested. The assay benefited from using the viability stain to reduce the number of false positives (data not shown). The allergen concentrations used in this study were standardized for comparative purposes and did not necessarily reflect the minimum concentration required for activation – another parameter that could be studied. Regardless, the fact that the assay was effective for all three allergens used suggests that the mBAT should be further evaluated for use with other allergenic proteins.

In summary, using sera from two adults with sesame allergy and one who also had walnut allergy, our inhibi-

tion immunoblots suggest that there is cross-reactivity between the walnut and sesame 11S globulin proteins, and this result was further confirmed using chicken antibodies raised against rJug r 4. We also found that rSes i 6 was able to activate basophils sensitized with sesame-allergic sera from seven of 11 different sesame-allergic patients who had *in vitro* IgE to rSes i 6, suggesting a potential contribution to clinical reactivity by the 11S globulin in these patients. The mBAT was also able to distinguish between potentially clinically relevant and irrelevant IgE reactivity towards walnut 11S globulin in two sesame-allergic sera that had IgE to both proteins. A weakness of our study is that the human sera were obtained from subjects who self-reported systemic reactions to walnut or sesame, rather than having undergone food challenges. With this weakness in mind, to strengthen the study, only sera donors who reported a history of reactions to sesame and were mBAT positive to our sesame extract (which may not contain all relevant allergens according to the recent work on sesame oleosins [22]) were chosen for further study. The same was true of the patients who reported walnut allergy. Another weakness of the present study is that sera IgE were not titred individually before use in the mBAT. Individual titration would allow for more controlled comparisons of the ability of polyclonal-specific IgE to activate basophils, which may reflect IgE affinity. Our lab is currently investigating the relationship between IgE affinity and clinically irrelevant IgE. Further study is thus warranted on the use of BATs in food allergy, which should facilitate studies of allergen cross-reactivity.

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