

## Contribution of Ara h 2 to peanut-specific, immunoglobulin E-mediated, cell activation

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### Clinical and Experimental Allergy

#### Summary

**Background** Ara h 2 is a potent peanut allergen but its contribution to the ability of a crude peanut extract (CPE) to cross-link IgE and activate mast cells has not been rigorously evaluated.

**Objective** To measure the contribution that Ara h 2 makes to the effector function of a CPE.

**Methods** Ara h 2 was specifically removed from a CPE as demonstrated by immunoblots, 2D gels, and an inhibitory ELISA. Functional assays of sham-treated and Ara h 2-depleted CPEs were performed with RBL SX-38 cells sensitized with IgE from highly peanut-allergic subjects and with naturally sensitized basophils.

**Results** Depletion of ~99% of the Ara h 2 from the CPE led to an increase in the concentration of the CPE necessary to give 50% of maximal degranulation (EC50) of the SX-38 cells following sensitization with sera that contain anti-Ara h 2 IgE. Assays with a pool of 10 sera showed a small but significant increase in the EC50 following depletion of Ara h 2 (1.65 ± 0.15-fold;  $P < 0.05$ ) and assays of seven individual sera showed a similar increase in the average EC50 (1.7 ± 0.2-fold;  $P < 0.02$ ). The percent of the anti-peanut IgE that binds Ara h 2 correlated with an increase in the EC50 of the CPE following depletion of Ara h 2 ( $r = 0.83$ ;  $P < 0.02$ ). On the other hand, data from three of these patients studied with a basophil histamine release assay did not show a significant effect of depletion of Ara h 2.

**Conclusion** Based on its ability to cross-link IgE effectively, Ara h 2 is clearly an important peanut allergen. Its ability to cross-link IgE effectively from a specific serum is related to the proportion of anti-Ara h 2 in that serum but Ara h 2 does not account for a majority of the effector activity of the CPE for any of the sera studied.

**Keywords** allergens, Ara h 2, IgE, mast cells, peanuts, RBL SX-38 cells

*Submitted 18 August 2006; revised 15 January 2007; accepted 1 February 2007*

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#### Introduction

Immediate hypersensitivity reactions to peanuts, *Arachis hypogaea*, are unusual in that they are much more prevalent and severe than for other legumes and tend to persist into adulthood [1]. Eight principal allergens of peanut (Ara h 1–Ara h 8) are accepted by the International Union of Immunological Societies, Allergen Nomenclature Sub-Committee [2–7]. Ara h 1 (63 kD), Ara h 2

(17–19 kD doublet) and Ara h 3 (60 kD) have been designated major peanut allergens based on the frequency of patients whose IgE binds to these proteins [8, 9]. Ara h 6, another important peanut allergen, is 59% homologous with Ara h 2 [10]. Lehmann et al. [11] have recently defined the protease-stable core of Ara h 2 and Ara h 6 and demonstrated resistance to proteolytic treatment. Significant efforts are underway to generate hypoallergenic peanuts and to design allergen-specific immunotherapy with genetically modified allergens [12–14].

Ara h 2 and Ara h 6 belong to the conglutin family of seed storage proteins, and are part of the 2S albumin

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family. Ara h 2, the subject of this report, constitutes approximately 0.1% of the peanut proteome [15, 16]. There are two main isoforms, Ara h 2.01 and Ara h 2.02, containing 10 independent IgE-binding epitopes stretching throughout the linear structure [3, 17]. The C-terminal region of Ara h 2 has homology with non-specific lipid-transfer proteins (nsLTPs) and with several other peanut proteins, specifically Ara h 6, Ara h 7, seed storage protein 1 (SSP-1), and seed storage protein 2 (SSP-2). Ara h 2 is a more potent allergen than is Ara h 1 in the RBL SX-38 cell assay, in basophil histamine release (BHR) assays, and upon percutaneous skin testing [18–20]. Ara h 2 is also more potent than Ara h 3 [20]. Ara h 6 and Ara h 2 are of similar potency [10]. Immunotherapy with recombinant Ara h 1, Ara h 2, and Ara h 3 confers protection against peanut challenge in a mouse model of peanut allergy [21]. However, it has not been proven that these major allergens account for a substantial part of the activity leading to mast cell activation mediated by human IgE.

One established way to test the hypothesis that an allergen accounts for a substantial part of the ability of an extract to effectively cross-link IgE leading to activation of mast cells is to remove the putative 'major' allergen by immunoprecipitation and demonstrate that the activity of the 'depleted' extract is reduced markedly. De Groot et al. [22] depleted an extract of cat dander of Fel d 1 (by 95%) with monoclonal and with polyclonal antibodies and found that the depleted extracts were 30–300 times less potent than the original extracts in BHR assays. Lombardero et al. [23] depleted an extract of olive pollen of the allergen Ole e 1 using monoclonal antibodies and found a large reduction in the allergenic activity as measured by BHR and by skin testing. A limitation of both of these studies is that the investigators did not establish that only the putative allergen (Fel d 1 or Ole e 1) was removed.

RBL SX-38 cells are rat basophilic leukaemia cells that stably express the  $\alpha$ ,  $\beta$  and  $\gamma$  chains of the human high affinity receptor for IgE, Fc $\epsilon$ RI [24]. This receptor confers these cells the important property that they can bind IgE from the sera of allergic individuals and can be activated in an allergen-specific manner [25]. Cells such as these can be easily used to determine the allergenic potency of extracts or specific allergens [19, 26, 27].

Because Ara h 2 appears to be more potent than Ara h 1 or Ara h 3, and because the combination of these allergens appears to account for the allergenicity of peanuts in a murine model of peanut allergy, we chose to test the hypothesis that Ara h 2 accounts for a substantial part of the ability of a crude peanut extract (CPE) to activate mast cells and basophils sensitized with IgE from a peanut-allergic patient. For this, we depleted a CPE of Ara h 2 and monitored the success of the depletion by immunoblot and 2D gels. We then tested the ability of this extract to cross-link human IgE in the RBL SX-38 cell and BHR assays.

## Materials and methods

### Subjects and sera

This study was approved by the Institutional Review Board at the University of Colorado Health Sciences Center. All subjects gave informed consent and/or assent. Otherwise healthy subjects between the ages of 4 and 70 with a strong history of severe systemic reactions to peanuts and anti-peanut IgE of at least 14 IU/mL were included in the study. These individuals have a >95% likelihood of having a systemic reaction to a blinded oral challenge with peanuts [28]. A pooled serum sample (serum pool #1) was prepared containing sera from 10 different patients based on the content of anti-peanut IgE. This was done to avoid undue influence of any one serum with large amounts of anti-peanut IgE. Serum pool #1 was used for most of the experiments but was exhausted before the manuscript was completed. For the final immunoblot using a polyvinylidene difluoride (PVDF) membrane, a second serum pool (serum pool #2) was prepared using a total of nine sera, eight of which were identical to those used for the original serum pool.

Twenty-nine subjects with strong histories for peanut sensitivity were evaluated, 16 met these criteria, and we had sufficient serum from 14 subjects. One patient (D47) was evaluated (total IgE = 761 IU/mL; anti-peanut IgE = 19 IU/mL) and then later excluded from the initial analysis of the effect of removing Ara h 2 from the CPE because there was no detectable anti-Ara h 2 IgE (<0.35 IU/mL). We reasoned that it was unfair to assess the effect of removing Ara h 2 with this serum in which the IgE was known not to bind Ara h 2. However, data from this patient are included in the final assessment of a correlation between the percent of IgE that binds Ara h 2 and the effect of removing Ara h 2 from the CPE. The characteristics of these 14 subjects and the composition of the serum pools are shown in Table 1.

### Reagents

Purification of Ara h 2 by anion and cation exchange chromatography was based on the protocol described by Maleki et al. [29] for isolation of Ara h 1 and is similar to that published by Sen et al. [30] except dithiothreitol (DTT) was added to decrease the amount of ammonium sulphate used during the extract fractionation and enable direct loading onto the anion exchange column. This is the same material as that used in a previous manuscript [19]. Briefly, a protein extract enriched with Ara h 2 was prepared by extraction of defatted raw peanut flour (Jumbo runner cultivar) at pH 8.3 in TE buffer [65 mM Tris-HCl, 5 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride (PMSF)] in presence of 200 mM NaCl, followed by ammonium

Table 1. Characteristics of patients and sera

Subject	Symptoms	IgE (IU/mL)	Anti-peanut IgE (kIU/L)	Anti-Ara h 1 IgE (kIU/L)	Anti-Ara h 2 IgE (kIU/L)	Anti-Ara h 2 (%)*
Serum pool <sup>†</sup>		291	46	14	15	33
Sera in serum pool						
D17	Resp./AE	180	44	5.5	11	25
D18 <sup>‡</sup>	Resp./Hives	161	64	19	15	23
D19	Resp./Hives	124	48	8.4	23	48
D26	Syst. Anaph.	373	90	48	36	40
D32	Resp./AE	298	64	18	18	28
D34	Syst. Anaph.	120	25	3.5	16	63
D42 <sup>‡</sup>	Resp./Hives	250	18	6.1	8.2	44
D43	Resp./Hives	386	30	8.6	7.4	25
D45	Hives	169	84	3.8	36	43
D48	AE/Resp.	277	132	92	27	20
D51 <sup>§</sup>	AE/Resp.	436	174	ND	ND	ND
Sera not in serum pool						
D29	AE/Resp.	106	26	4	15	58
D47	Resp./Hives	761	19	<0.35	<0.35	0
D51	AE/Resp.	436	174	37	63	36
D53	Resp./Hives	1560	158	31	54	34
Mean ± SD		376 ± 370	77 ± 57	22 ± 25	25 ± 17	35 ± 16

\*Anti-Ara h 2 IgE as a percent of the anti-peanut IgE.

<sup>†</sup>Sera were pooled so that each serum contributed the same amount of anti-peanut IgE based on ImmunoCap<sup>®</sup> data. Therefore the IgE values for the serum pool are not an average of values for the individual sera.

<sup>‡</sup>Present in serum pool #1 only.

<sup>§</sup>Present in serum pool #2 only.

sulphate precipitation at 14% saturation. After high-speed centrifugation, Ara h 2 in the supernatant was precipitated by increasing the ammonium sulphate from 14% to 22% saturation. The 22% precipitate was resuspended in TE buffer by sonication, cleared by centrifugation, and then loaded onto a DEAE weak anion exchange column (Bio-Rad, Hercules, CA, USA) and eluted with a linear gradient of NaCl (100–300 mM). Fractions were analysed by SDS-PAGE with a Coomassie blue staining. Early fractions containing Ara h 2 (doublet) alone (no Ara h 1 contamination) were pooled, concentrated, buffer exchanged to distilled water (pH 7.0 with drops of 6 N NaOH) containing 1 mM DTT, and then lyophilized.

#### Crude peanut extract

Twenty grams of fresh raw Georgia green peanuts, a kind gift from the Golden Peanut Company, Ashburn, GA, USA were frozen in liquid nitrogen and finely ground with a mortar and pestle. Following extensive defatting with diethyl ether, the dried peanut flour was resuspended in 100 mL of ice-cold buffer [150 mM NaCl, 50 mM Tris, pH 7.4 with protease inhibitors (Roche, Nutley, NJ, USA complete, EDTA-free)] and mixed overnight at 4 °C. The extract [25 mg/mL (BCA, Pierce, Rockford, IL, USA)] was clarified by centrifugation (10 000 g, 30 min), sterile filtered, and frozen in aliquots at –70 °C.

#### Anti-peptide antibodies

Two peptide epitopes (13–14 amino acids each) of Ara h 2 [GenBank sequence AAN77576 (gi:26245477)] were chosen in consultation with Dr Shing-Erh Yen (Invitrogen, Carlsbad, CA, USA) based on uniqueness, antigenicity, surface probability, flexibility, and an antigenic index [31, 32]. The antibody that was most useful was directed against the C-terminal sequence, CDLEVESGGRDRY, which consists of amino acids 160–172 of Ara h 2.02 (GenBank sequence AAN77576 and SwissProt accession number, Q8GV20) and is also the C-terminus of Ara h 2.01 [3, 33]. The second peptide was DRRDPYSPSPYDR, which consists of amino acids 79–91 of Ara h 2.02 (above), and includes the immunodominant IgE-binding epitope DPYSPS that is also present in Ara h 2.01 [3, 33]. Two rabbits were immunized with peptide-KLH conjugates of both peptides [regular antibody service with a combination immunization strategy (Invitrogen)]. Antibodies were affinity purified on peptide columns and eluted with 3 M KSCN. The yields were 5–12 mg. Both antibodies from each rabbit identified a 17–19 kDa doublet on immunoblots (data not shown). The affinity-purified anti-Ara h 2 peptide antibodies were individually conjugated to activated agarose beads as per the manufacturer's instructions (Pierce ProFound<sup>™</sup> Co-Immunoprecipitation Kit, Rockford, IL, USA). Only the antibodies against the Ara h 2 amino acids #160–172 bound strongly enough to

Ara h 2 in solution to deplete CPE of Ara h 2. This antibody was used throughout the experiments to follow. Preimmune IgG was isolated on protein G and covalently linked to sepharose as described above. Anti-peptide antibodies against Ara h 1 (amino acid #599–612; KE-SPEKEDQEEE NQ; Swiss-Prot, Q547W5), Ara h 3 (amino acid # 90–103; EEPHTQGRRSQSQR; Swiss-Prot, O82580), and Ara h 6 (amino acid # 42–59; EQEQYD-SYNGFSTRSSDQ; Swiss-Prot, Q9SQG5) were raised and used for immunoblots.

#### *Depletion of Ara h 2 from a crude peanut extract*

CPE was diluted to 1 mg/mL in coupling buffer (Pierce) and 800  $\mu$ L was incubated with the anti-Ara h 2-conjugated agarose column containing  $\sim$ 5 mg of antibody (overnight, 4 °C). The eluate was then collected and stored at –80 °C. A control column was constructed as outlined above, except that pre-immune rabbit IgG (purified on protein G) was used to generate sham-treated CPE. The columns were regenerated by treatment with 0.1 M glycine, pH 2.5, a total of three times before binding activity was lost.

#### *Two dimensional gel electrophoresis*

All gel equipment and supplies were obtained from Amersham Biosciences/GE Healthcare (Piscataway, NJ, USA) unless otherwise noted. Fifty micrograms of protein was minimally labelled with Cy3 (Ara h 2-depleted CPE) and Cy5 (sham-treated CPE) CyDyes (4 °C, 30 min). Cy3- and Cy5-labelled samples were mixed together and brought up to a volume of 450  $\mu$ L in standard rehydration buffer. The sample was passively rehydrated overnight onto a 24 cm immobilized pH gradient strip (IPG 3–10 NL) and isoelectric focusing (IPGphor apparatus, Amersham Biosciences/GE Healthcare) was then performed according to the manufacturer's recommendations. Reduction and alkylation were carried out on-strip using DTT and iodoacetamide, respectively. Strips were then applied to a precast 8–16% SDS-PAGE gel (Jule Inc., Milford, CT, USA) for electrophoresis (Ettan DALT 12 electrophoresis unit, 20 W/gel, 25 °C). Labelled proteins were visualized by scanning the gels at 100  $\mu$ m resolution (Typhoon 9400 scanner, Amersham Biosciences/GE Healthcare).

#### *MALDI-TOF MS protein identification*

Protein spots of interest were excised from the gel using the Ettan robotic picker and placed into a micro-well plate for in-gel digestion using the Ettan digester. Plugs were washed twice with 100  $\mu$ L of 50 mM  $\text{NH}_4\text{HCO}_3$ /50% methanol for 5 min, once with 100  $\mu$ L of 75% acetonitrile for 10 min and twice with 100  $\mu$ L of 100% acetonitrile for 10 min. The gel plugs were dried at room temperature for 50 min and

then trypsin (Promega, San Luis Obispo, CA, USA) was added to each well (10  $\mu$ L, 10  $\mu$ g/ $\mu$ L in 20 mM  $\text{NH}_4\text{HCO}_3$ ) and digestion proceeded at room temperature for 16 h.

All digests were analysed by MALDI-TOF MS (Voyager DE-PRO, Applied Biosystems, Foster City, CA, USA) using peptide mass fingerprinting as described previously [34, 35]. Spectra were collected over the range  $m/z$  500–5000. No cleanup of digests was performed. Peptide mass fingerprints were internally calibrated to monoisotopic trypsin autolysis peaks ( $m/z$  = 515.33, 842.51, 1045.56, 2211.11). Spectra were processed using ProTS Data (Steamboat Springs, CO, USA) to generate centroided peak lists that were submitted to MS-Fit program of the Protein Prospector Suite (v. 4.21.3, UCSF Mass Spectrometry Facility, San Francisco, CA, USA) for database searching. Spectral processing included defining baseline, noise and signal-to-noise ratio as well as monoisotopic peak selection. A signal-to-noise ratio in ProTS data of  $>4$  was required for inclusion in the peak list. Database searches were conducted using the non-redundant protein database (NCBI, database version 05/07/2006) and the Swissprot database (version 2007.05.02). Other settings in ProTS included peak amplitude set at 100, peak width 250, and chemical noise factor 1.5. Key parameters included the following: peptide error tolerance was set at  $\pm$  60 p.p.m., fixed modification of carbamidomethylation of cysteine side chains, and variable modification of hydroxylation of P, oxidation of M, and protein N-terminus acetylated. Searches were not constrained by pI or molecular weight.

#### *Immunoblots*

1D gel immunoblots: 30  $\mu$ g of sham treated or Ara h 2-depleted CPE in standard (reducing) sample buffer were run on 4–20% polyacrylamide gels and transferred onto nitrocellulose with 20 v overnight at 4 °C in transfer buffer (25 mM Tris/192 mM glycine/20% methanol). The nitrocellulose was blocked for 2 h in 3% milk powder in tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and probed with rabbit anti-peptide antibodies to Ara h 1, Ara h 2 (#78–92), Ara h 3, and Ara h 6 at dilutions of 1 : 100–400. The nitrocellulose was developed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1 : 20 000), followed by an enhanced chemiluminescence reagent (Amersham) for 10–60 s.

2D gel immunoblots: 450  $\mu$ g of untreated CPE was run on a 2D gel, transferred to nitrocellulose as described above, probed with human serum, and developed with HRP-murine anti-human IgE (1 : 3000) (Bio-Rad). A replicate gel was transferred onto a PVDF membrane as suggested by Boldt et al. [36]. For the blot onto the PVDF membrane, all reagents and settings were the same as for nitrocellulose, except the HRP-murine anti-human IgE was diluted to 1 : 30 000 to avoid a high background.

### ImmunoCap<sup>®</sup> assay with Ara h 2

ImmunoCap<sup>®</sup> assays for Ara h 1 and Ara h 2 were performed as described previously [19].

### Enzyme linked immunoglobulin assay inhibition assay

Fifty microlitres of purified Ara h 2 (5 µg/mL in 0.1 M Na carbonate buffer, pH 9.6) was adhered to microtitre wells at 4 °C overnight. The plate was then washed with 1 × phosphate-buffered saline (PBS)/0.05% Tween 20 (PBST) and then blocked for 2 h at RT with PBS containing 3% non-fat milk powder (Bio-Rad). During the blocking time, affinity-purified rabbit anti-Ara h 2 (amino acids: 145–157), diluted 1 : 100 000 (final concentration = 10 ng/mL) in PBST with 3% non-fat milk, was incubated with varying concentrations (0–1.0 µg/mL) of purified Ara h 2, sham-treated CPE or Ara h 2-depleted CPE. The anti-Ara h 2 with or without inhibitors was added to the wells containing adhered Ara h 2 for 1 h at room temperature. Following extensive washing the plate was developed with horseradish peroxidase labelled mouse anti-rabbit IgG and tetra methyl benzidine (TMB) (Sigma, St Louis, MO, USA). A standard curve was generated based on inhibition with purified Ara h 2 (0.001–1.0 µg/mL). We found that low levels of Ara h 2 (<0.01 µg/mL) were difficult to measure in the depleted extract, presumably due to interference by cross-reactive proteins (see the immunoblot in Fig. 1). For this reason, a secondary curve was generated based on the sham-depleted extract and this was used to calculate the Ara h 2 content in the depleted extract (data not shown).

### RBL SX-38 cell assay

The sensitivity and specificity of the RBL SX-38 cell assay have been previously described [19, 25]. RBL SX-38 cells sensitized with IgE from patients with undetectable anti-peanut IgE are not activated when exposed to a CPE [25]. RBL SX-38 cells were grown and the functional assays performed with <sup>3</sup>H-5HT (tritium-labelled, 5-hydroxytryptamine or serotonin) as described previously [19]. Labelled and sensitized RBL SX-38 cells were triggered with anti-IgE (3 µg/mL), CPE (250 ng/mL), depleted CPE or sham-treated CPE (0.25–250 ng/mL). Percent release of serotonin was calculated as described previously [24, 25].

### Basophil histamine release

BHR was performed with unwashed basophils from peanut-allergic subjects using the Histamine Elisa Kit (Beckman Coulter, Miami, FL, USA). Depleted CPE and sham-treated CPE were diluted (0.025–250 ng/mL) in the histamine release buffer supplied by the manufacturer.

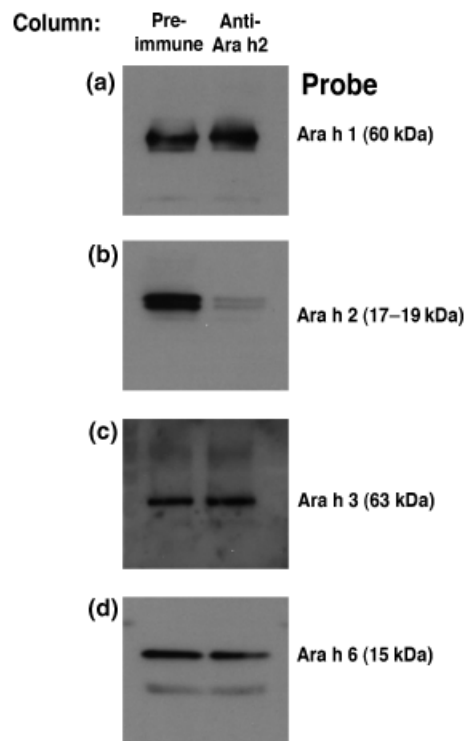


Fig. 1. Depletion of Ara h 2 but not Ara h 1 from crude peanut extract (CPE) as demonstrated on immunoblot. Five hundred nanograms of sham-treated CPE or Ara h 2 depleted-CPE were run on a 4–20% polyacrylamide gel and transferred onto nitrocellulose. The peanut proteins were then probed with either affinity-purified anti-Ara h 1, amino acids #599–612 (a); affinity-purified anti-Ara h 2, amino acids #79–91 (b); affinity-purified anti-Ara h 3, amino acids #90–103 (c); or with affinity-purified anti-Ara h 6, amino acids #42–59 (d). Development with enhanced chemiluminescence was 10–30 s. This was repeated with similar results.

Spontaneous degranulation was subtracted from total degranulation with CPE to give the 'net degranulation' and data are presented as percent histamine release. Basophils were also stimulated after washing in the histamine release buffer. This caused a marginal increase in the background but with a very similar dose response for both depleted and sham-treated CPE (data not shown).

### Determination of EC<sub>50</sub> and calculation of the allergenic potency of the Ara h 2-depleted crude peanut extract

The EC<sub>50</sub> of an extract or allergen is the dose giving 50% of the degranulation seen with an optimal amount of CPE. To determine EC<sub>50</sub> accurately, the effect of five half-log dilutions was studied. The values for the concentrations of the extracts were log-transformed and non-linear regression was performed using a second order polynomial equation. The intercept on the abscissa was determined for a value of 50% on the ordinate. Computer-generated best-fit lines are shown in each figure. An increase in the EC<sub>50</sub> represents a decrease in potency [26].

### Reproducibility

The RBL SX-38 cell assay was performed with a single serum sample (D19) on three separate 96-well plates on three separate days over a period of several months to determine the inter- and intra-assay coefficient of variation (CV) ( $SD/mean \times 100$ ) [37]. The intra-assay CV for determination of the EC 50 of a CPE was  $29 \pm 6\%$  ( $n = 3$ ) and the inter-assay CV ( $SD/mean \times 100$ ) was 105% (data not shown). Thus, within an assay, we can detect differences of EC50 in the range of 30%, and values for EC50 across separate experiments therefore vary, on the average, by a factor of 2. For each serum, the comparisons of Ara h 2-depleted and sham-treated CPE were done in the same assay.

### Statistical analysis

Statistical comparisons were all two tailed. *P*-values  $< 0.05$  were considered to be statistically significant. To carry out comparisons among experiments, release of serotonin was expressed as a percentage of that seen with optimal doses of the original CPE in the same assay. Data from Ara h 2-depleted and sham-depleted extracts were compared with paired *t*-tests. Deviations of ratios from the predicted value of 1.0 were analysed with one-sample *t*-tests. Spearman's rank correlation test was used to assess correlations. All figures, best-fit lines, EC50 values and statistical data were generated with GraphPad Prism version 4.0b (GraphPad Software, San Diego CA, USA).

## Results

### Successful depletion of Ara h 2

Incubation of CPE with the anti-Ara h 2 agarose column successfully removed  $99 \pm 1\%$  (mean  $\pm$  SD,  $n = 3$  comparisons) of Ara h 2 (Fig. 1b) without depleting Ara h 1 (Fig. 1a), Ara h 3 (Fig. 1c) or Ara h 6 (Fig. 1d). The extent of removal of Ara h 2 was also confirmed by 2D gel analysis (Fig. 2). The lower images (Fig. 2) compare the sham-treated (left panel) and Ara h 2-depleted (right panel) CPEs on a single gel. The set of four spots in the region 17–19 kDa (indicated by a box) are present in the sham-treated extract, but are almost completely absent in the Ara h 2-depleted extract. These protein spots co-migrated by molecular size with purified Ara h 2 although the DTT-treated Ara h 2 had a slightly more acidic pI (data not shown). Each spot was excised from the gel, digested with trypsin, and peptide mass maps were generated by MALDI-TOF mass spectrometry. All four spots were identified as isoforms of Ara h 2. At least 20 peptides covering  $> 75\%$  of the sequences were involved in making each match (data not shown). The only other peanut protein depleted by this process was an unidentified protein of  $\sim 50$  kDa that does not correspond to a known peanut

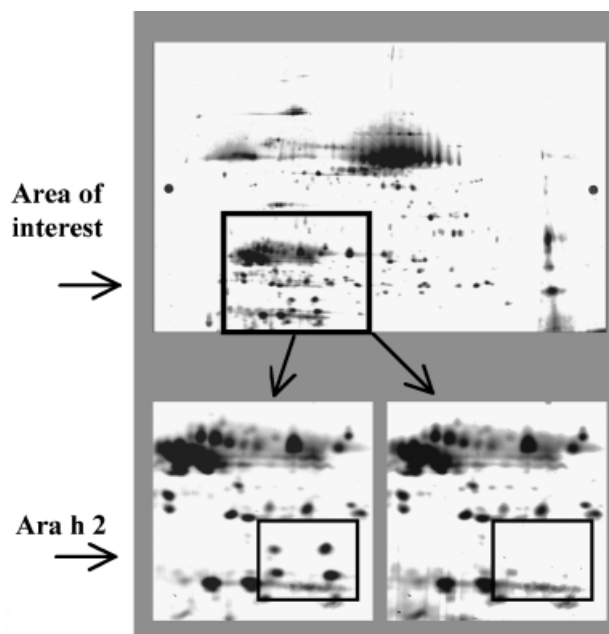


Fig. 2. Selective depletion of Ara h 2 from crude peanut extract (CPE) demonstrated on two dimensional gel. Fifty micrograms of sham-treated and Ara h 2-depleted CPE were minimally labelled with Cy3 and Cy5 CyDyes, respectively, and run together. Top, the full gel for CPE is shown for reference. The image on the lower left (sham-treated CPE) was scanned at 570 nm (Cy3) and the image on the lower right (Ara h 2-depleted CPE) was scanned at 670 nm (Cy5). The 4 spots ( $\sim 17$ – $19$  kDa) that are identified by a rectangle (lower left panel) co-migrate with purified Ara h 2 (data not shown) and are almost completely absent from the depleted CPE (lower right panel).

allergen (data not shown). We also confirmed the extent of depletion of Ara h 2 with the inhibitory ELISA described in 'Materials and methods'. Based on this assay, we determined that the sham-treated extract contained  $0.18 \pm 0.070$  (mean  $\pm$  range of two measurements)  $\mu\text{g}$  of Ara h 2 per 100  $\mu\text{g}$  of CPE and estimated that the Ara h 2-depleted extract contained 0.005  $\mu\text{g}$  of Ara h 2 per 100  $\mu\text{g}$  of CPE ( $\sim 97\%$  depleted) (data not shown). We believe that this is an underestimate of the extent of depletion due to presence of other cross-reacting proteins. The data for the sham-treated CPE are similar to the report of Schmitt et al. [16] in which Ara h 2 constituted approximately 0.1% of the protein content of several different peanut extracts.

### Ara h 2 depleted crude peanut extract is less potent than the sham-treated extract

Depletion of 99% of Ara h 2 from the CPE has only a small effect on the ability of the CPE to cross-link IgE effectively from peanut-allergic patients as measured in the RBL SX-38 cell assay (Fig. 3). The dose response of sham-treated CPE is very similar to that of Ara h 2-depleted CPE either for cells sensitized with sera pooled from 10 donors (serum pool #1) (Fig. 3a) or when sensitized with sera from

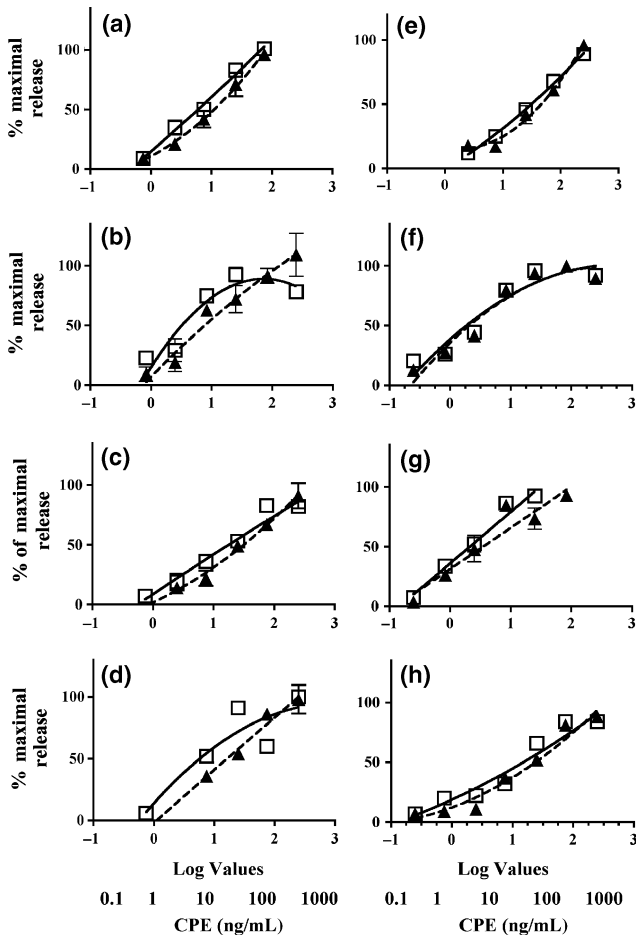


Fig. 3. Effect of depletion of Ara h 2 in the RBL SX-38 cell assay of IgE cross-linking. RBL SX-38 cells were sensitized with sera from peanut-allergic subjects and stimulated with untreated crude peanut extract (CPE) (not shown), sham-treated CPE ( $\square$ , solid lines) or Ara h 2-depleted CPE ( $\blacktriangle$ , dashed lines). Spontaneous degranulation (buffer) was subtracted from total degranulation to give the 'net degranulation' as described in 'Materials and methods'. In these experiments, the background degranulation was  $18 \pm 3\%$  ( $n=6$ ; mean  $\pm$  SEM) and following the maximal dose of untreated CPE, the total degranulation was  $64 \pm 5\%$ . After subtracting the background for each experiment, this gives a net release was  $47 \pm 4\%$ . With sham-treated CPE and with the Ara h 2-depleted CPE, the net releases were  $44 \pm 4$  and  $42 \pm 7\%$  respectively. In this figure, net degranulation for all data points is expressed as percentage of maximal degranulation within each assay. All assays were performed in triplicate and data points are mean  $\pm$  SD. If standard error bars are not visible, the error was smaller than the symbol. Data using the following sera are shown: (a) the serum pool, (b) D19, (c) D26, (d) D29, (e) D32, (f) D48, (g) D51, (h) D53. Two additional replicates with the serum pool and D47 are not shown.

individual donors (Figs 3b–h). Three of the sera shown in Fig. 3 were part of serum pool #1 and 4 were not. EC50 values with the sham-treated and Ara h 2-depleted CPEs were determined for serum pool #1 and for seven individual sera, four of which were part of the serum pool (Table 2). All the sera in the serum pool and all the seven individual sera used to calculate the effect of removing Ara h 2 from the CPE contained anti-Ara h 2 IgE

(7–63 kIU/L) (Table 1). For the serum pool, the EC50 with the Ara h 2-depleted CPE is  $1.6 \pm 0.15$ -fold more than that seen with the sham-treated CPE ( $n=3$ ;  $P < 0.05$ ). Similarly, looking at seven individual sera, the EC50 with the Ara h 2-depleted CPE is  $1.7 \pm 0.21$ -fold more than that seen with the sham-treated CPE ( $n=7$ ;  $P < 0.02$ ). An assay was also performed with the serum (D47) that had no detectable anti-Ara h 2 IgE. The EC50 with the sham-treated extract was 54 ng/mL and for the depleted extract was 47 ng/mL. This change of  $-13\%$  is within the error of the assay and is compatible with no change due to depletion of Ara h 2 as should be the case as there is no IgE that binds to Ara h 2. Similar experiments with basophils (naturally sensitized with IgE) from three of the peanut-allergic donors showed no consistent effect of removal of 99% of the Ara h 2 from the CPE (Figs 4a–c and Table 2;  $P = \text{NS}$ ).

#### Correlation between the RBL SX-38 cell assay and the ImmunoCap<sup>®</sup> assay

It is reasonable to ask whether the amount of IgE that binds to Ara h 2 in the ImmunoCap assay predicts the

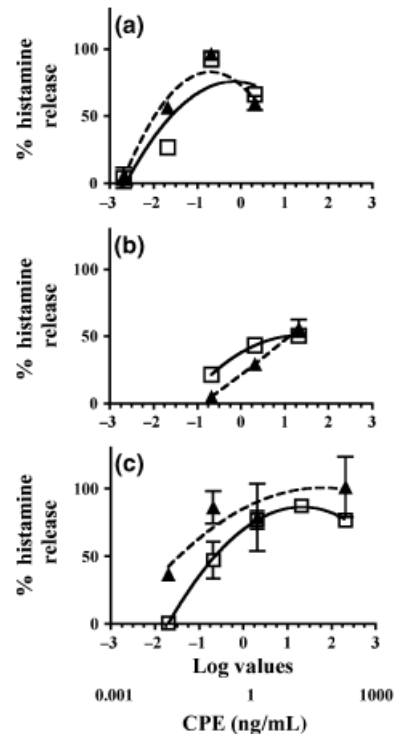


Fig. 4. Functional assay of IgE cross-linking on basophils from peanut-allergic subjects. Basophils in heparinized blood were stimulated with buffer, sham-treated crude peanut extract (CPE) ( $\square$ , solid lines), or Ara h 2-depleted-CPE ( $\blacktriangle$ , dashed lines). All assays were performed in triplicate and data points are mean  $\pm$  SD. If standard error bars are not visible, the error was smaller than the symbol. In these experiments, the background degranulation was  $9 \pm 4\%$  ( $n=3$ ; mean  $\pm$  SEM). Data using basophils from the following subjects are shown: (a) D19, (b) D51, (c) D53.

Table 2. Effective concentration 50 (EC50)

Sera	RBL SX-38 cells			Basophils		
	EC50 (ng/mL)		Fold change in EC50 (Ara h 2-depleted CPE/sham-treated CPE)	EC50 (ng/mL)		Fold change in EC50 (Ara h 2-depleted CPE/sham-treated CPE)
	Sham-treated CPE	Ara h 2-depleted CPE		Sham-treated CPE	Ara h 2-depleted CPE	
<b>Serum pool*</b>						
Assay #1	14	19	1.4	ND	ND	
Assay #2	28	49	1.8	ND	ND	
Assay #3	6.0	11	1.8	ND	ND	
95% CI			1.0–2.3			
Mean ± SEM	16 ± 6	26 ± 12	1.65 ± 0.15			
P-value*			< 0.05			
P-value†			< 0.04			
<b>Individual sera with anti-Ara h 2‡</b>						
D19	6.2	8.9	1.4	0.025	0.011	0.44
D26	18	31	1.7	ND	ND	
D29	5.7	16	2.8	ND	ND	
D32	34	44	1.3	ND	ND	
D48	2.4	2.9	1.2	ND	ND	
D51	2.2	4.0	1.8	11	13	1.18
D53	15	24	1.6	0.23	0.035	0.15
95% CI			1.2–2.2			
Mean ± SEM	12 ± 4	19 ± 5	1.7 ± 0.21	3.8 ± 3.7	7.5 ± 4.3	0.59 ± 0.53
P-value*			< 0.02			NS
P-value†			< 0.004		NS	
<b>Individual serum with no anti-Ara h 2‡</b>						
D47	54	47	0.87	ND	ND	

Paired *t*-test comparing log values of sham treated and Ara h 2 depleted.

\*Serum pool includes D19, D26, D32, D48 and 6 other sera (not shown individually).

†Mean is different from 1.0; One sample *t*-test.

‡This serum did not contain any anti-Ara h 2 specific IgE and is not included in the analysis of the effect of depleting Ara h 2.

CPE, crude peanut extract; CI, confidence interval.

importance of Ara h 2 in the CPE for individual sera studied in the RBL SX-38 cell assay. As can be seen in Fig. 5, there is a strong correlation ( $r=0.83$ ;  $P < 0.02$ ;  $n = 8$ ) between the percent of anti-peanut IgE that binds to Ara h 2 in the ImmunoCap assay (abscissa) and the fold-increase in the CPE for that serum following depletion of Ara h 2 (ordinate). The data with the pooled serum (anti-Ara h 2 IgE = 33%) of the anti-peanut IgE and shift in EC50 due to depletion of Ara h 2 of 1.65-fold are not plotted but fall near the best-fit line.

#### Many potential allergens are seen on a two dimensional immunoblot

Because depletion of Ara h 2 from the CPE has only a minor impact on the allergenicity of the extract, it is reasonable to ask how large is the repertoire of potential peanut allergens? For this reason, a 2D immunoblot was performed with the serum pools (Figs 6a and d) and with serum from one individual patient (Fig. 6b) to identify other candidate allergens. A control blot (Fig. 6c) was probed with serum from a peanut-tolerant patient with

allergic rhinitis (total IgE = 960 IU/mL; anti-peanut IgE = <0.35 IU/mL). Ara h 2 is clearly visible in Figs. 6a, b and d, although the density of binding of IgE to Ara h 2 is dwarfed by that of other proteins especially in the blots using nitrocellulose (Figs 6a and b). Previous work has shown that purified Ara h 2 binds IgE less well on immunoblots than does Ara h 1 [19] and this is recapitulated here. The 2D blot probed with the control serum showed no specific spots after a 5-min exposure (Fig. 6c). The 2D gel transferred onto a PVDF membrane instead of nitrocellulose showed a somewhat different pattern with decreased detection of higher molecular weight allergens and enhanced detection of lower molecular weight allergens (Fig. 6d). Thus, many peanut proteins specifically bind IgE (Figs 6a, b and d) and, based on these findings, could account for the majority of the effector activity of peanuts.

#### Discussion

Ara h 1, Ara h 2, and Ara h 3 have been identified as the major peanut allergens based on the large numbers of

patients who have IgE that bind to proteins migrating at 63, 17–19 kD, and 60 kD, respectively [1, 9, 15, 38]. Ara h 6 has been shown to be an important peanut allergen with a potency similar to that of Ara h 2 [10]. The definition of what constitutes a ‘major’ peanut allergen is still evolving [39, 40].

Peanut-allergic patients have a wide range of total and peanut-specific IgE and the data obtained with these patients who have high anti-peanut IgE (>14 kU/L) may not pertain to all peanut-sensitive subjects. However, there is no current reason to believe that this will not be the case.

Although the ability of an allergen to generate an IgE response in susceptible patients and to bind IgE in *in vitro* assays is important, we, and others, have focused on the ability of allergens to cross-link IgE and lead to activation of mast cells and basophils as an important measure of allergenicity [19, 20, 25, 26]. In addition to binding IgE, critical determinants of this function of allergens are the number of IgE-binding epitopes on the allergen and the allergen’s affinity for IgE [41–43]. Based on functional assays, Ara h 2 is more active at cross-linking IgE than are Ara h 1 or Ara h 3 and is similar to Ara h 6 [10, 19, 20].

The ability of allergens to cross-link IgE bound to FcεRI can be studied with cell-based assays such as the RBL SX-38 cells or human basophils sensitized *in vivo* or *ex vivo* with anti-peanut IgE [25, 44]. The RBL SX-38 cell assay has advantages over the BHR assay in that it does not require fresh cells from a patient for each experiment. The BHR assay has advantages over the RBL SX-38 cell assay in that the entire system is from the affected patient. Koppleman *et al.* were able to test Ara h 1, 2, and 3 with skin test titrations in allergic subjects [20]. Unfortunately, in the United States, skin testing such as this requires an investigational new drug (IND) application for each extract or purified protein to be tested. This makes skin testing impractical in the United States.

Ara h 2 is clearly a potent and important allergen as it constitutes 0.1–0.2% of the peanut proteome and it binds a substantial amount of IgE from peanut-allergic patients (Table 1). These experiments were designed to test the hypothesis that Ara h 2 is also responsible for a substantial portion of the ability of a CPE to cross-link IgE from a peanut-allergic subject. We did this by quantitatively and specifically removing Ara h 2 from CPEs by immunodepletion and then assessing the ability of the depleted extract to cross-link IgE/IgE receptor complexes as measured by cell activation. Sham-treated and Ara h 2-depleted extracts were tested for their ability to cross-link human anti-peanut IgE on RBL SX-38 cells sensitized with IgE from peanut allergic subjects or on naturally sensitized basophils from peanut-allergic subjects. Removing ~99% of the Ara h 2 (Figs 2 and 3) has a modest effect in the RBL SX-38 cell assay that is variable among sera (Fig. 4) and no consistent effect in the BHR assay

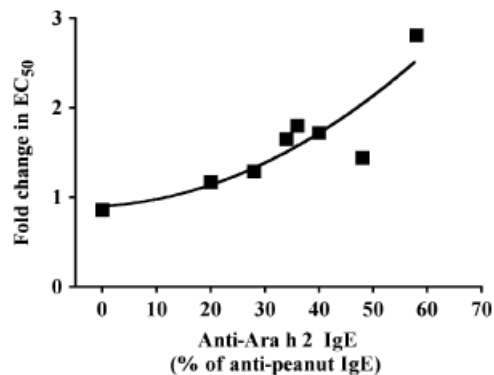


Fig. 5. Correlation between the percent of anti-peanut IgE that binds to Ara h 2 and the effect of depletion of Ara h 2 on the RBL SX-38 cell assay. For eight individual sera, the percent of anti-peanut IgE that binds to Ara h 2 from Table 1 is plotted on the abscissa and fold increase in EC50 of the extract for that serum when Ara h 2 is removed from the crude peanut extract is plotted on the ordinate (Table 2). Data from patient D47 (no binding of Ara h 2) is included. Spearman’s correlation coefficient = 0.83;  $P < 0.02$ . A best-fit line was generated using a polynomial equation  $Y = A + B^X + C^{X^2}$  and is shown.

(Fig. 5). Although the coefficient of variation of the BHR assay is reported to be 30%, the lack of an effect on the BHR assay probably reflects its lack of precision in our hands [45].

These data are very different from those of de Groot *et al.* [22], who found that depletion of 95% of Fel d 1 by monoclonal antibodies reduced the allergenic potency by a factor of 30–40. Our data are also very different from those of Lombardero *et al.* [23], who found that depletion of 99.5% of Ole e 1 from an extract of *Olea europaea* resulted in a shift in the BHR dose–response of 125 times.

The quantitative contribution of Ara h 2 to the potency of the CPE is related to the quantitative change in the EC50, but determining the specific contribution from the available data is not straightforward. It is reasonable to argue that, if depletion of 99% of Ara h 2 resulted in a complete diminution of allergenicity for a given serum, the EC50 would shift from its initial value to infinity and we would say that the entire potency of the CPE is due to Ara h 2 for that serum. On the other hand, if depletion of Ara h 2 from the CPE led to a 10-fold increase in the EC50 (the depleted extract was 10-fold less potent), we propose that, in this situation, Ara h 2 would account for 90% of the activity of the extract for that specific serum. Similarly, if the change in EC50 were threefold (~1/2 log), Ara h 2 could be said to account for ~45% (1/2 of 90%) of the activity. Our data showing only a  $1.7 \pm 0.2$ -fold increase in the EC50 due to removing Ara h 2 suggest that the contribution of Ara h 2 to the allergenic potency of the CPE is, on the average, in the 20% range, although variable among our subjects. Using our approach to determine the contribution of an allergen to the potency of an extract, we argue that the de Groot data demonstrate

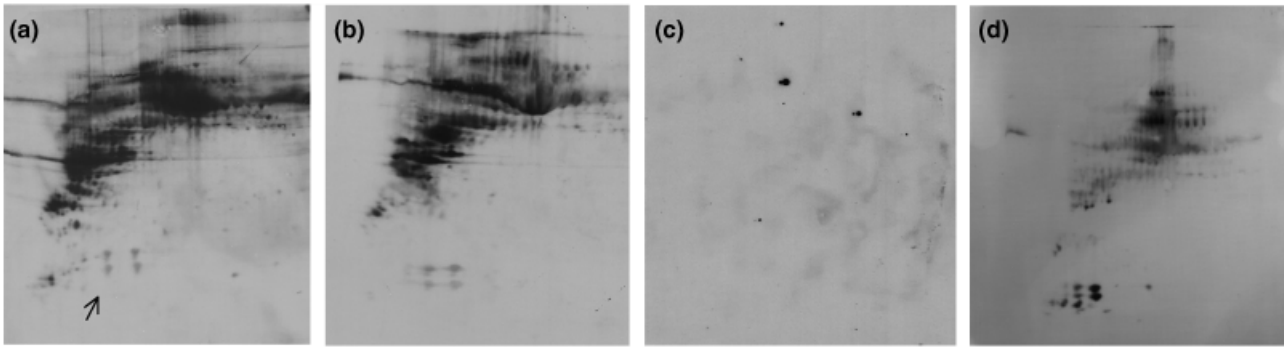


Fig. 6. Two-dimensional (2D) immunoblot of crude peanut extract. Four hundred and fifty micrograms of a crude peanut extract was separated on a 2D gel, transferred onto nitrocellulose (a–c) or a polyvinylidene difluoride (PVDF) membrane (d), washed, and developed with horseradish peroxidase-murine anti-human IgE as described. All images shown were after a 5-min exposure with enhanced chemiluminescence. (a) Probed overnight with a 1 : 10 dilution of serum pool #1 (final IgE = 23 IU/mL; final anti-peanut IgE = 4.3 IU/mL). (b) Probed overnight with a 1 : 20 dilution of serum from a peanut-allergic subject (D26), (final IgE = 19 IU/mL; final anti-peanut IgE = 4.5 IU/mL). (c) A control blot was probed overnight with a 1 : 30 dilution of serum from a peanut-tolerant, highly grass pollen-sensitive patient with anti-peanut IgE < 0.35 kU/L (final IgE = 32 IU/mL; final anti-grass IgE = 3.1 IU/mL). (d) 2D gel transferred onto a PVDF membrane and probed with a 1 : 10 dilution of a second batch of pooled serum (pool #2; see 'Materials and methods') (final IgE = 24 IU/mL; final anti-peanut IgE = 3.9 IU/mL).

that Fel d 1 accounted for ~95% of the allergenic potency of their cat dander extract and the Lombardero data demonstrate that Ole e 1 accounts for ~99% of the allergenic potency of the olive pollen extract.

One potential limitation of this study is the variability of these biologic assays. However, in our hands, the intra-assay coefficient of variation of the RBL SX-38 cell assay for determination of the EC<sub>50</sub> is  $29 \pm 6\%$  (data not shown), makes this a very sensitive assay of cell activation. We found that depletion of 99% of Ara h 2 from the CPE leads to a  $1.65 \pm 0.15$ -fold increase ( $P < 0.05$ ) in the EC<sub>50</sub> in the RBL SX-38 cell assay using a serum pool and a  $1.7 \pm 0.2$ -fold increase ( $P < 0.02$ ) using seven individual sera, four of which were in the serum pool. In contrast to the findings with the SX-38 cells, depletion of Ara h 2 had no detectable effect on the BHR assay. Even though BHR has been used to assess allergenic extracts that have been depleted of specific allergens and can easily detect a log change in activity, the BHR assay is not optimal for making this type of comparison [22, 23].

Another potential limitation of this study is the use of raw and not roasted peanuts. We decided to use extracts of raw peanuts for two reasons. First, peanut extracts used clinically for skin testing and peanut flour used for food challenges are made with raw peanuts. Second, roasted peanuts have been reported to bind more IgE and the Ara h 2 trypsin inhibition activity is increased [46–48]. This type of experiment would be much more complicated with roasted peanuts where adducts of Ara h 2 are formed with other peanut proteins, making immunodepletion technically much more challenging.

Because depletion of Ara h 2 from a CPE has a small but significant effect on the activity of the CPE in these cellular assays, it is reasonable to conclude that Ara h 2 is an important allergen and, because it is more potent than Ara h

1 and Ara h 3, it is likely more important. However, Ara h 2, by itself does not account for a substantial part of the ability of our CPE to cross-link IgE from our cohort of peanut-allergic patients. Not surprisingly, the importance of Ara h 2 appears to be greater in those patients with IgE that binds the most Ara h 2 (Fig. 5). It is possible that, if we were able to identify patients whose IgE binds large amounts of Ara h 2 and conversely small amounts of Ara h 1, 3, and 6, the contribution of Ara h 2 would be more significant.

However, based on our finding on 2D immunoblot (Fig. 6), there are a large number of potential peanut allergens. By this logic, some other allergens may synergize with Ara h 2, may act independently, or may act in concert with other allergens to account for the activity in CPEs. An excellent candidate that may account for a substantial part for this remaining activity is the related peanut allergen, Ara h 6, a protein of 15 kD. It is possible that the reactivity to peanut allergens may be due to a group of allergens working in concert and the nature of these peanut allergens may vary from patient to patient. An alternative to this idea is that Ara h 2 is a major allergen responsible for a great deal of the observed cross-linking, but when it is removed, other allergens with homologous linear and/or 3D epitopes become more active.

#### Acknowledgements

The authors thank Mr Mike Simpson for technical assistance, Dr Pamela Wolfe for statistical advice and Ms Andrea T. Dreskin and Ms June K. Inuzuka for help in preparing the figures.

This work was supported by an NIH grant, AI052164-01, to Dr Dreskin and institutional funds, UCHSC and NJMRC.

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