

Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil–histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen

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Summary

Background A number of allergenic proteins in peanut has been described and the relative importance of these allergens is yet to be determined.

Objectives We have investigated the relevance of previously identified peanut allergens in well-characterized peanut-allergic patients by *in vitro*, *ex vivo* and *in vivo* assays.

Methods Thirty-two adult peanut-allergic patients were included based on careful and standardized patient history and the presence of peanut-specific IgE. The diagnosis peanut allergy was confirmed using double-blind placebo-controlled food challenges in 23 patients. Major peanut allergens Ara h1, Ara h2 and Ara h3 were purified from peanuts using ion-exchange chromatography. IgE immunoblotting was performed and IgE-cross-linking capacity was examined by measuring histamine release (HR) after incubating patient basophils as well as passively sensitized basophils with several dilutions of the allergens. Intracutaneous tests (ICTs) using 10-fold dilution steps of the purified allergens and crude peanut extract were performed.

Results Ara h2 was recognized most frequently (26 out of 32) in all tests and induced both positive skin tests and basophil degranulation at low concentrations, whereas Ara h1 and Ara h3 were recognized less frequently and reacted only at 100-fold higher concentrations as analysed with HR and intracutaneous testing (ICT). Next to the three tested allergens, proteins with molecular weights of somewhat smaller than 15 kDa were identified as a IgE-binding proteins on immunoblot in the majority of the patients (20 out of 32).

Conclusion We conclude that Ara h2 is, for our patient group, the most important peanut allergen, and that previously unidentified peanut proteins with molecular weights of somewhat smaller than 15 kDa may be important allergens as well. ICT in combination with basophil–HR and IgE immunoblotting provides insight in the patient specificity towards the individual peanut allergens.

Keywords allergens, Ara h1, Ara h2, Ara h3, basophils, histamine release, immunoblot, peanut, purification, skin test

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Introduction

Peanut allergy is one of the most severe food allergies due to its persistency and the life-threatening character [1]. The prevalence of peanut allergy in the United Kingdom is estimated to be about 0.5% [2] and in the USA, the prevalence of peanut and tree nut allergy is 1.1% [3], representing a main health concern. Doses as low as 100 µg of peanut protein may already provoke symptoms [4, 5], indicating that accidental ingestion of minute traces of peanut can endanger the life of peanut-allergic patients. The nature

of the allergenic compounds in peanuts has been studied extensively in the last decade. Ara h1 was identified as a major peanut allergen [6, 7] and has gained a considerable amount of attention in recent years. Ara h1 was purified and characterized on a molecular level. It appears to be a 63 kDa glycoprotein, with isoforms having different isoelectric points [8], that is present in peanut as a trimer [9, 10]. The amino acid sequence of Ara h1 [11] was elucidated after cloning the gene. Subsequently, synthetic peptides based on this sequence were synthesized and 23 IgE-binding epitopes were mapped [12]. However, the recombinant Ara h1 appeared to contain a 86 amino acid N-terminal sequence that is not found in Ara h1 purified from peanuts [7]. Interestingly, this sequence contained two immunodominant IgE epitopes [12]. Ara h2 was identified as a second important peanut allergen, although the relative importance of Ara h2

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compared with Ara h1 is disputed [7, 13, 14]. Cloning techniques have revealed the complete amino acid sequence of Ara h2 [15]. Ara h3 was in first instance identified by Eigenmann et al. [16] as a 14 kDa peanut protein, but cloning its gene revealed a protein of approximately 60 kDa identified as peanut glycinin with IgE-binding sites throughout the gene product [17]. Meanwhile, Kleber-Janke described an identical peanut allergen, and confusingly the official name given by the IUIS Allergen Nomenclature Sub-Committee was Ara h4. The differences in molecular weight described for Ara h3 suggest that the gene product in peanut is stored in a processed form, as is the case for Ara h1. Another aspect that hampers the research of peanut allergens is that a number of different polypeptides are derived from one gene product. This results in a set of proteins with probably different molecular characteristics that are referred to only one name. For example, Ara h3 purified from peanut consists of a set of polypeptides with molecular weights ranging from 14 to 45 kDa [18].

The gold standard in diagnostics of food allergy is the double-blind placebo-controlled food challenge (DBPCFC). However, this test is expensive and laborious and patient safety can be a dilemma. Several *in vitro*, *ex vivo* and *in vivo* diagnostics tests can be performed to address the implication of IgE-peanut-allergen interactions. In patient serum CAP/RAST or ELISA can demonstrate IgE antibodies specific for an allergen, assays in which the allergen is coupled to a solid phase. By immunoblotting allergens are separated on basis of their molecular weight and subsequently tested for their IgE-binding properties. Functional IgE-binding tests that also take into account the cross-linking of the IgE receptor are basophil degranulation assays and skin prick or intracutaneous tests (ICTs).

The aim of the current study was to assess the relevance of the peanut-derived purified allergens Ara h1, Ara h2 and Ara h3 by immunoblotting, histamine release (HR) assays and skin tests for 32 well-characterized adult peanut-allergic patients.

Methods

Peanut protein extraction

Peanuts (*Arachis hypogea*) from the Runner cultivar (Cargill, Dawson, GA, USA) were generously provided by Imko Nut Products (Doetinchem, the Netherlands) and were stored at 10 °C until use. Peanuts were ground and protein extracts were made by mixing 25 g ground peanut with 250 mL 20 mM Tris buffer (pH 7.2). After 2 h stirring at room temperature, the aqueous fraction was collected by centrifugation (3000 g, at room temperature for 30 min). The aqueous phase was subsequently centrifuged (10 000 g at room temperature for 30 min) to remove residual traces of fat and insoluble particles. Protein concentrations were determined using Bradford analysis with BSA as a standard. Reducing SDS-PAGE from the extracts showed protein bands between 14 and approximately 100 kDa (not shown). Extracts (crude peanut extract, CPE) contained on average 26 mg/mL protein and were stored at -20 °C.

Purification of Ara h1, Ara h2 and Ara h3

Ara h1 and Ara h2 were purified, generally according to previously described methods [6, 7, 13], and Ara h3 was purified as described recently [18] using ion-exchange chromatography. Purified allergens were analysed on SDS-PAGE and individual protein bands were N-terminally sequenced using automated Edmann degradation in order to confirm their identity [18]. Ara h1 migrates as a single protein band at 63 kDa, and Ara h2 migrates as a doublet at 17–20 kDa. Ara h3 is composed of a set of protein bands with molecular weights ranging from 14 to 45 kDa. In the last step of the purification, Ara h1 and Ara h3 are both bound to the column and elute successively. This implies that traces of Ara h1 may be present in the Ara h3 preparation and vice versa. Based on gel densitometry, the contamination levels were estimated less than 1%. Because Ara h2 is base line separated from Ara h1 and Ara h3, the contamination of Ara h2 with Ara h1 and Ara h3 is much less, and estimated to be less than 0.01% [18]. For the use in BHR and ICT, Ara h1, Ara h2, Ara h3 and CPE were sterilized in the following way. Preparations were applied on sterile disposable PD-10 gel permeation columns (Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with sterile phosphate-buffered saline (PBS). Protein containing fractions were collected and filtered through a sterile 0.22 µm filter. The resulting Ara h1, Ara h2, Ara h3 and CPE preparations were diluted with sterile PBS to a concentration of 200 µg/mL, and stored in aliquots of 100 µL at -20 °C.

Patient characteristics

Thirty-two adult peanut-allergic patients (median age 26.5 years, range 17–41 years, 23 women and nine men) were included in this study (Table 1) [5]. All patients had a convincing history of acute peanut-related symptoms. Symptoms (by history) were classified according to the scheme of Muller [19] (Table 1), which was originally designed for classification of insect sting-induced-allergic reactions. All patients had a positive skin prick test (SPT) using a commercial peanut-extract (ALK Albello, Frederiksberg, Denmark) of $\geq 2+$ except in one patient where SPTs could not be performed. IgE antibodies directed against peanut were determined using CAP-system FEIA (Pharmacia & Upjohn, Uppsala, Sweden) and were positive in all patients except one (patient no. 4). DBPCFCs were performed in 23 patients as described elsewhere [5]. Serum for identifying allergen recognition patterns was obtained from all patients and stored in aliquots at -20 °C. This study was reviewed and approved by the ethical committee of the University Medical Centre Utrecht. All patients gave written informed consent before enrolment in the study.

Intracutaneous testing

A selection of patients who were willing to cooperate in further studies was included for intracutaneous testing (ICT). ICT using purified natural Ara h1, Ara h2, Ara h3 and CPE were performed in 11 out of 32 patients according to Dreborg and Frew [20]. Antihistamines were discontinued 1 week prior to the skin tests and no topical corticosteroids were used. Skin tests were performed on the flexor aspect of the patients arm

Table 1. SPT, peanut-specific IgE and total IgE in patient sera

Patient number	SPT peanut (ALK)	Cap peanut (IU/mL)	Total IgE (IU/mL)	Peanut-related symptoms (by history)	Muller classification	MPD (mg peanut protein) as determined using DBPCFC
2	3+	0.43	304	Oa,le	1	
4	3+	39	154	Oa,gu,rc	2	1000
8	4+	33	1904	Oa,le,gi	2	
9	4+	<0.35	26	Oa	1	10
14	4+	4	761	Oa,gi	2	
17	3+	12	1170	Oa,ae,dys	3	
18	3+	39	352	Oa,le,gi,gu,dys	3	
19	4+	99	348	Oa,gu,dys	3	0.1
20	4+	5.2	804	Oa,gi,dys	3	
21	4+	66	>5000	Oa	1	
22	4+	77	4382	Oa,gi,lu	2	10
23	4+	2.97	164	Oa,gi	2	0.1
26	3+	2.93	1355	Oa,gi,lu	2	1
30	4+	3.08	196	Oa,gi,dys	3	3
32	3+	2.85	349	Oa	1	1000
34	4+	42	>5000	Oa	1	1000
35	ND	ND	7170	Oa,le,dys	3	
37	4+	22	>5000	Oa	1	10
38	4+	75	211	Oa,le,dys	3	
40	4+	>100	521	Oa,le,gi,gu,dys,rc	3	0.3
42	3+	15.3	1010	Oa,le,gi,dys,gu,lu	3	3
43	3+	5.9	174	Oa	1	1000
44	3+	26.7	94	Oa,gi,dys	3	10
45	3+	0.37	196	Oa	1	30
47	4+	4.2	1155	Oa,le,gu	2	100
48	4+	>100	>5000	Oa,le,gi,gu	2	30
49	3+	4.2	1187	Oa,gi	2	30
50	4+	13.5	4240	Oa,gi	2	0.3
52	4+	24.8	432	Oa,le,gi,gu,dys	3	0.3
53	4+	44	113	Oa,le,gi,dys	3	0.1
54	4+	94	487	Oa,le,gi,gu,dys	3	1
69	2+	1.56	216	Oa,gi,rc	2	100

Oa, oral allergy syndrome; le, larynx oedema; gi, gastro-intestinal symptoms; gu, generalized urticaria; ae, angio-edema; dys, dyspnea; rc, rhinoconjunctivitis; lu, local urticaria.

Muller classification: 1, symptoms of the oral cavity and skin symptoms; 2, gastro-intestinal symptoms and/or laryngeal oedema; 3, dyspnoea; 4, cardiovascular symptoms like decrease in blood pressure with additional collapse. SPT, skin prick test; DBPCFC, double-blind placebo-controlled food challenge.

and were located 5 cm apart. Histamine dihydrochloride and the dilution buffer served as a positive and negative control, respectively. Sterile stock solutions containing 200 µg/mL allergen were used for preparing serial dilutions using a diluent containing NaHCO₃ 5 mg, phenol 5 mg, human serum albumin (HSA) 0.3 mg and NaCl 2.5 mg/mL (ALK Albello). Several 10-fold dilution steps were used starting with a concentration varying between 2×10^{-7} and 2×10^{-5} µg/mL up to a maximum concentration varying between 2×10^{-4} and 2×10^{-1} µg/mL, depending on the subjects response. An interval of 15 min was allowed between each dose increment. The results are expressed as the amount of injected allergen in picograms that is able to induce a histamine-equivalent ICT (HEIC = mean diameter of the allergen weal divided by the mean diameter of the histamine weal) score of ≥ 0.25 . When one of the allergens gave a positive response, the two other allergens were tested only in two higher concentrations (100-fold) since possible contamination of one allergen with the other (less than 1%) may interfere at higher concentrations. Three non-atopic and three atopic individuals were used as

controls. The atopic individuals were also sensitized for grass pollen, cat dander and house dust mite. ICTs in these control persons with Ara h1, Ara h2, Ara h3 and CPE up to a maximum concentration of 0.2 µg/mL showed negligible responses.

SDS-PAGE and immunoblotting

SDS-PAGE was performed essentially according to Laemmli [21] using a BioRad Mini Protean II system (BioRad, Hercules, CA, USA) with 15% acrylamide gels (15 × 10 cm). Pre-stained molecular weight markers with molecular weights of 14.3, 21.5, 30, 46, 66, 97.4 and 220 kDa were used as reference. Samples were mixed in a 1:1 ratio with 63 mM Tris buffer (pH 6.8) containing 1% dithiotreitol (DTT), 2% SDS, 0.01% bromophenol blue and 20% (v/v) glycerol and were subsequently boiled for 5 min. The proteins amounts of the lanes with purified allergens were chosen such that the amount of purified allergen corresponded to the amount of the particular allergen present in the CPE lane. Gels were stained with Coomassie brilliant blue R-250 dissolved in

destaining solution (10% HAc (v/v), 5% methanol (v/v) in water). After destaining, gels were scanned with an ImageMaster DTS (Pharmacia, Uppsala, Sweden). To study the immuno-reactivity of the proteins, SDS-PAGE gels were prepared as described above and the separated proteins were transferred to polyvinylidene difluoride sheets (Immobilon-P, Millipore Corp., Bedford, MA, USA) generally as described by Towbin et al. [22]. Membranes were blocked with 3% BSA in wash buffer (50 mM Tris, pH 7.5, containing 0.1% BSA and 0.1% Tween 20) for 1 h at room temperature. Patient serum was diluted 1/120 in wash buffer and applied on the membrane (overnight at room temperature). Bound IgE was detected using a peroxidase-conjugated anti-human IgE monoclonal antibody (kindly provided by Dr Jonas Lidholm, Pharmacia Diagnostics, Uppsala, Sweden) and a subsequent staining reaction for peroxidase activity using the enhanced chemiluminescence (ECL) technique. Between steps, blot membranes were washed five times with wash buffer. Blots were dried and scanned with an ImageMaster DTS. Non-specific binding of the anti-IgE antibody conjugate was measured in a similar blotting procedure omitting the incubation step with patient sera and appeared to be negligible.

Collection of mononuclear cells from peripheral blood

Blood (20–50 mL) was obtained in sodium citrate-containing tubes from peanut-allergic individuals and non-peanut-allergic controls by venapuncture. Mononuclear cells were isolated after separating the blood on an isotonic Percoll solution with a specific gravity of 1.078 g/cm³ [23]. Mononuclear cells were resuspended in incubation medium (132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.2 mM potassium phosphate, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) HSA, pH 7.4) and kept on ice until further use. The cells were counted electronically (Sysmex, Microcell Counter, Kobe, Japan) and after one wash resuspended in incubation medium supplemented with 1 mM CaCl₂ in a final concentration between 2.5×10^6 and 5×10^6 /mL.

Sensitization of peripheral blood mononuclear cell (PBMC) with patient serum

To sensitize donor's basophils with serum, first autologous IgE was dissociated from the basophils in the PBMC population by acid elution [24]. PBMC (100×10^6 – 150×10^6) were washed in NaCl (0.9%) and the cell pellet was resuspended in 3 mL lactic acid (0.09% (v/v)). After 2 min at 20 °C the reaction was stopped by adding 7 mL incubation medium. After a 2-fold washing in incubation medium, the cell pellet was resuspended in 1 mL incubation medium. To sensitize the basophils with IgE, 500 µL of this cell suspension was diluted with 2.5 mL incubation medium supplemented with 20% (v/v) patient serum, 10 µg/mL heparin and 4 mM EDTA and incubated for 90 min at 37 °C. Subsequently, the cells were washed twice and resuspended for 30 min at 37 °C in incubation medium supplemented with 1 mM CaCl₂. Dissociation and subsequent sensibilization were controlled by flow cytometric analysis using FITC-labelled goat anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Each individual patient serum was tested on at least three different basophil donors.

Basophil–HR

Basophil–HR was performed as previously described [25] both with basophils from the blood of allergic patients and with basophils that were obtained from control donors sensitized with patient serum. In brief, basophils were stimulated with 10-fold serial dilutions of CPE, Ara h1, Ara h2 and Ara h3. To check for donor-specific basophil releasability [489] both IgE-mediated and C5a-induced HR were included. Anti-IgE was used in concentrations 1 : 30, 1 : 300 and 1 : 3000 (covering the bell-shaped response curve of basophils after anti-IgE) and C5a was added in the optimal concentration of 10 nM. The amount of HR in the cell-free supernatants was determined by fluorometric analysis as described by Siraganian [26]. Unstimulated basophil preparations that did release more than 10% of their histamine content were not analysed. To correct for donor-specific IgE-mediated HR [27], the threshold levels of the allergen-induced release were calculated at the concentration allergen that induced 50% of the maximal release induced by anti-IgE. The analysis of basophils from anti-IgE non-responding donors releasing less than 10% of their histamine content after anti-IgE stimulus were discarded from this analysis.

Results

IgE specificity analysed by immunoblotting

Patient IgE reactivity towards Ara h1, Ara h2, Ara h3 and CPE was analysed by immunoblotting and Fig. 1 shows some examples of the immunoblot experiments. Patient 23 very specifically reacts with Ara h2, both in the Ara h2 lane and the CPE lane. There is no IgE binding to Ara h1, Ara h3 or any other peanut protein. Patient 23 is judged as negative for Ara h1 and Ara h3 and positive (++) for Ara h2. Patient 54 reacts strongly with Ara h1, Ara h2, Ara h3, as well as with other proteins between 5 and 100 kDa. Patient 45 shows only vague bands (doublet) at approximately 20 kDa in CPE. This is indicative for Ara h2, but reactivity towards purified Ara h2 was not observed, possibly due to a somewhat lower amount of purified Ara h2 compared with the Ara h2 content in CPE. Using another serum dilution (30 instead of 120) purified Ara h2 as well as Ara h2 in CPE were recognized. For patient 45 IgE binding to Ara h2 was considered slightly positive (+), whereas there was no binding towards Ara h1, Ara h3, or other protein bands.

All patient sera were analysed in immunoblotting and the results are summarized in Table 2. The majority of the patients (26 out of 32) reacted with Ara h2, whereas 14 of our 32 patients reacted with Ara h1 and 12 of our 32 patients reacted with Ara h3. For a substantial number of patients, IgE binding to proteins somewhat smaller than 15 kDa were visible (20 out of 32 patients), as well as a band around 30 kDa (six out of 32 patients). A total of four out of 32 patients did not react with any of the peanut protein bands on immunoblot. Remarkably, all patients who demonstrated IgE binding to Ara h3 also demonstrated IgE binding to Ara h2 and Ara h1 and in most of those cases, the reactivity towards Ara h2 was more pronounced. Patients 18 and 69 selectively reacted with Ara h1 and in 12 patients Ara h2 was the only allergen recognized. Ara h3 was in none of the patients a uniquely recognized peanut allergen.

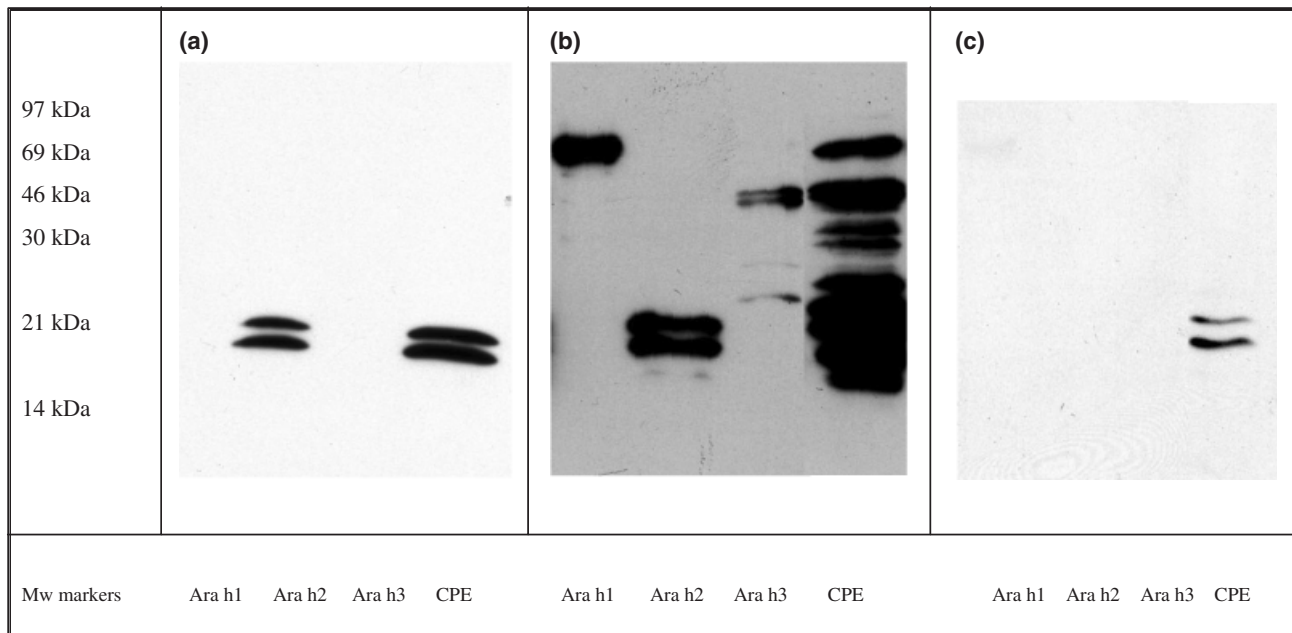


Fig. 1. Example of Western blotting experiment. (a) Patient 23, (b) patient 54, (c) patient 45.

Table 2. Summary of results from IgE immunoblot

Patient number	Ara h1	Ara h2	Ara h3	Additional band observed in CPE (kDa)
2	—	—	—	
4	—	—	—	
8	—	+	+	30, <15
9	—	+	—	
14	—	+	—	<15
17	—	+	—	<15
18	+	—	—	30
19	+	+	+	<15
20	—	+	—	<15
21	+	+++	++	<15
22	+	+++	+++	
23	—	++	—	
26	—	+	—	<15
30	—	+	—	<15
32	—	—	—	
34	—	+	++	30, <15
35	++	++	++	<15
37	—	+	—	<15
38	+++	+++	+++	5–100
40	+++	+++	+++	5–100
42	+	++	—	<15
43	—	—	—	
44	++	++	+	<15
45	—	+	—	
47	—	+	—	
48	+	+++	++	<15
49	—	+	—	
50	—	++	—	<15
52	+	++	—	<15
53	++	++	+	<15
54	+++	+++	+++	5–100
69	++	—	—	

CPE, crude peanut extract.

Basophil–HR

To analyse the IgE-cross-linking capacities of the purified allergens, patient basophils were stimulated with the peanut allergens and degranulation was enumerated by measurement of histamine released. Figure 2a shows the titration curves of a representative patient. As is clear for this patient, CPE is a potent stimulus. Concentration range was set following a previous paper indicating that concentrations above 10 µg/mL were causing allergen non-specific degranulation [28]. The concentration of Ara h2 required for half-maximal release is for basophils of patient 54 <0.1 ng/mL. For Ara h1 and Ara h3 these values are 1 and 10 ng/mL, respectively. To rule out non-specific effects due to the priming status of the patient basophils that might result in IgE-independent stimulation of the basophils by compounds in CPE, we subsequently analysed basophils from control donors who were passively sensitized with serum from the peanut-allergic individuals. Figure 2b shows the HR after peanut allergens were incubated with basophils of four different (not peanut allergic) donors sensitized with serum of patient 54. This indirect HR showed sensitivities for the peanut allergens comparable with the direct stimulation of basophils of patient 54 depicted in Fig. 2a. The immunoblotting (Table 2) demonstrated that several allergic patients did not show IgE binding to the peanut allergens. To rule out that this was due to possible insensitivity of the technique, we selected both positive and negative sera for the cross-linking potencies in the basophil–HR test. A total of 10 sera of peanut-allergic patients were evaluated in this manner. As is clear from Table 3 the sera not responsive in the immunoblots did also not induce HR. Again Ara h2 was the most potent peanut allergen.

Skin tests using purified peanut allergens and crude peanut

To achieve a more exact volume of allergen dilution presented to the skin, ICT was chosen rather than SPT. In 11 out of 32

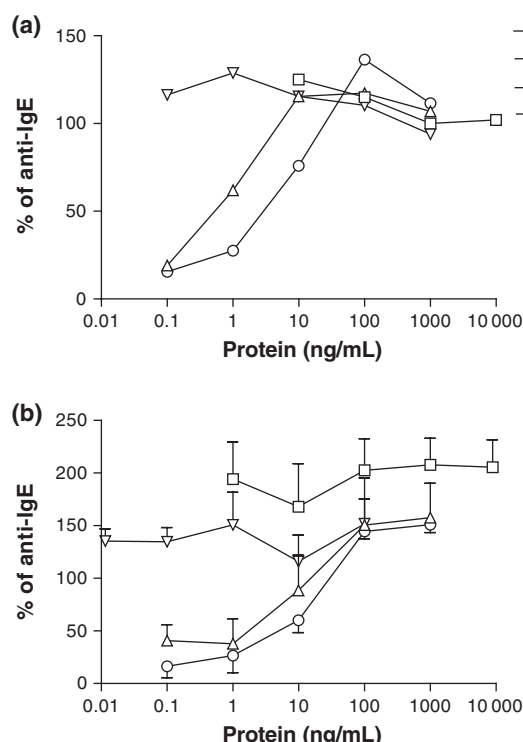


Fig. 2. Basophil-histamine release (HR). (a) Direct HR from patient basophils (patient 54). (b) HR from basophils from non-peanut-allergic donors loaded with patient serum (patient 54). Titration curves for Ara h1, Ara h2, Ara h3 and crude peanut extract (CPE) are shown.

Table 3. HR with purified peanut allergens

Patient number	HR threshold (ng) for histamine >50% of anti-IgE-induced HR			
	Ara h1	Ara h2	Ara h3	CPE
4	∞	∞	∞	∞
18	1	0.1	∞	<1
19	10	<0.01	10	1
23	∞	∞	∞	∞
32	∞	∞	∞	∞
40	1	<0.01	<0.1	<1
45	∞	∞	∞	∞
47	∞	∞	∞	1000
49	∞	∞	∞	∞
54	10	<0.01	100	<1

Indirect HR assays with the serum of 10 patients. The lowest concentrations of purified allergens able to induce more than 50% of the anti-IgE-induced HR are listed. Each serum was analysed independently on the basophils of two to four different donors. The average net percentage of histamine released after anti-IgE was for the individual serum used, 4: 15.9%, 18: 20.8%, 19: 15.4%, 23: 19.0%, 32: 16.1%, 40: 21.5%, 45: 15.4%, 47: 23.6%, 49: 14.5% and 54: 12.0%. ∞ indicates that no response was found at the highest concentration tested. HR, histamine release; CPE, crude peanut extract.

patients ICTs were performed using Ara h1, Ara h2, Ara h3 and CPE. This yielded positive responses to one or more of the three purified peanut allergens in all patients. However, the sensitivity among patients differed a 1000-fold (Table 4). Nine patients were most sensitive to Ara h2, two patients to Ara h1. Only the latter two were monosensitized, the others also recognized Ara h1 and/or Ara h3 next to Ara h2 but to a

Table 4. ICT with purified allergens

Patient number	ICT threshold (pg) for HEIC ≥2+			
	Ara h1	Ara h2	Ara h3	CPE
4	10 000	10	10 000	1000
9	10 000	100	∞	1000
18	1	∞	∞	1
19	1	0.1	10	1
23	1000	10	∞	10
40	10	1	10	1
45	1000	100	∞	100
47	1000	10	∞	100
49	1000	10	10 000	1000
69	1000	∞	∞	100 000

ICT, intracutaneous test; HEIC, histamine-equivalent ICT; CPE, crude peanut extract.

lesser extent. CPE yielded positive responses in all patients at a dose equal to or slightly higher than the 'most sensitive' allergen, indicating that the relevant allergen is present in CPE in a lower concentration.

Discussion

We have investigated the *in vivo*, *ex vivo* and *in vitro* relevance of previously identified peanut allergens in a large group of well-characterized peanut-allergic patients. For this, we used purified Ara h1, Ara h2 and Ara h3 derived from peanuts, as described previously [18]. Based on this patient population we demonstrate that Ara h2 is the most frequently recognized, as well as the most potent peanut allergen.

With regard to the characteristics of peanut allergens, there are some controversies, mainly based on differences between purified peanut protein and recombinant allergens. These are most probably due to incomplete or different post-translational processing or different glycosylation of the recombinant vs. the purified protein. Differences between purified and recombinant allergens have been shown before for pollen allergens in diagnosis of pollen allergies [29]. We chose to examine the allergens purified from peanut to reflect as much as possible their natural characteristics. A disadvantage of purified vs. recombinant material might be the relative impurity of the purified material. Although some contamination of Ara h1 in the Ara h3 fraction is unavoidable, it is clear for instance from the HR of patient 18 (Table 4), as well as immunoblotting data depicted in Fig. 2b, that the contamination of Ara h1 in Ara h3 is less than 1%.

Relative importance of the individual peanut allergens

In order to assess the relative importance of the individual peanut allergens, we reconsider Table 2 with the immunoblot results. Next to information about the individual allergens Ara h1, Ara h2 and Ara h3, immunoblot data also reveal information about other proteins in CPE and about sub-units within Ara h3. We show that the allergen recognized most frequently in peanuts is Ara h2, in 26 out of 32 cases. The relative recognition of Ara h1 and Ara h2 was studied using immunoblotting earlier [6, 7, 13, 14] and was recently

reviewed [30]. The data reported here are in good accordance with previous studies from European populations [7, 14], but the first report on Ara h1 obtained from a North American population described a strikingly higher incidence of Ara h1-specific IgE in peanut-allergic patients [6]. Recently, it was shown that this difference is not caused by variation in the allergen content of consumed peanuts since all current peanut cultivars contain similar levels of Ara h1 and Ara h2 [30]. It is therefore likely that North Americans and Europeans encounter the same peanut allergens. A recent publication by Sen et al. [31] described that the immunodominant IgE-binding epitopes in Ara h2 are very resistant to enzymatic digestion. This might explain further the relative importance of Ara h2, compared with the other peanut allergens. The importance of Ara h2 in HR and ICT was not only illustrated by the percentage of positive sera, but also in the potency of Ara h2 at relatively low concentrations in these tests. Our results that Ara h2 is more often recognized, and a more potent peanut allergen compared with Ara h1 are supported by the work of Palmer et al. [32] who demonstrated that Ara h2 was the most potent peanut allergen as assayed with HR from a transformed rat mucosal mast cell line sensitized with patient sera.

Ara h1 is recognized in 14 out of 32 patients, and in 12 of them there is also reactivity towards Ara h2 with Ara h2 being the more potent allergen as demonstrated in HR and ICT tests. There are two patients (18 and 69) who exclusively recognize Ara h1 and they are remarkable since they developed peanut allergy some time after they became allergic to pea. In a recent study, we demonstrated that Ara h1 and pea vicilin are clinically relevant cross-reacting allergens for these two patients [33]. The hypothesis that sensitization to peanut Ara h1 originated from a pea vicilin sensitization may explain the low number of patients who exclusively react with Ara h1. The peanut allergen specificity of these patients was confirmed using ICT and the unique Ara h1 specificity was confirmed, indicating that in these patients Ara h1 is the only *in vivo* relevant peanut allergen. Up to now, we do not have a suitable explanation why a weak reactivity towards Ara h2 was found with the HR test. However, sensitivity for Ara h2 was at least 10-fold lower than for the other Ara h2 responding sera.

There is only limited information in the literature about the importance of Ara h3, and the data that are available deal with either the 14 kDa N-terminal fragment [16, 34] or with recombinant Ara h3 [14, 17]. From our population, 12 out of 32 patients recognized Ara h3, in agreement with reports on recombinant Ara h3 [14, 17]. In a recent paper from our group the biochemical characterization and IgE-binding properties of Ara h3 were described [18] and IgE binding was demonstrated on both the N-terminal fragments (45, 42, 14 kDa), as well as on the internal and C-terminal fragments (28, 25, 16 kDa). Whereas no sequence homology between Ara h3 and Ara h1 or Ara h2 is found, IgE towards Ara h3 cannot cross-react with Ara h1 or Ara h2. Therefore, it is remarkable that recognition of Ara h3 was always found in conjunction with sensitization to other peanut allergens. For the patients tested, this was confirmed in the HR test and ICT indicating the *in vivo* relevance of Ara h3. We can only speculate on the reason why sensitization and *in vivo* activity of Ara h3 is exclusively observed in patients who are also

reactive for Ara h1 or Ara h2. Upon an allergic reaction, the function of the intestinal epithelium is distorted leading to increased permeability [35] with increased macromolecular passage, most likely via the paracellular route [36]. Knippels et al. [37] showed that substantial levels of β -lactoglobulin were present in sera of ovalbumin-allergic rats challenged with ovalbumin in the presence of β -lactoglobulin. It may be that patients allergic to Ara h1 or Ara h2 become sensitized for Ara h3 due to an increased paracellular uptake during an allergic reaction towards peanut. We are currently verifying this secondary sensitization hypothesis towards Ara h3 in young peanut-allergic children who should have less sensitization incidence towards Ara h3.

For four of our peanut-allergic patients (numbers 2, 4, 32 and 43) no IgE binding could be detected in the immunoblot and in the HR two of these four were tested and also no reaction towards any of the peanut allergens was found. This might be due to a lower sensitivity of these *in vitro* tests. Remarkably, patients 4, 32 and 43 were also tested in the DBPCFC and responded only after 1000 mg (Table 1). This is equal to a total of six peanuts, which indicates a mild allergy. In general there may be a trend that high MPD levels are associated with low IgE levels as measured on immunoblot, but the number of exceptions is too large to speak of a correlation.

The majority of the patients recognized an allergen somewhat smaller than 15 kDa (20 out of 32 cases). Sometimes more than one band in this molecular weight range is recognized. It is interesting to note that these protein bands are not present in any of the three tested purified peanut allergen preparations. Based on the estimated molecular weight, these proteins may be Ara h5, Ara h6 or Ara h7. Their molecular weights have been calculated via their cloned genes and are 14, 14.5 and 15.8 kDa, respectively [14]. It is not likely that the presently found IgE-binding protein is a fragment of Ara h3, although they have similar molecular weights (14 kDa), [16, 18, 34], because the majority of patients recognizing this protein do not recognize Ara h3 (Table 2). Because of the high recognition rate, and because of the often intense reaction on blot, this allergen might be another relevant allergen in peanut. We now have preliminary data to suggest that this IgE-binding protein is Ara h6 (manuscript in preparation).

This is the first study reporting on the combined *in vitro* and *in vivo* reactivity of individual peanut-derived allergens. We conclude that Ara h2 is for the present group of patients the most important peanut allergen. Next to the purified allergens tested, a protein with a molecular weight somewhat smaller than 15 kDa is recognized by the majority of the patients, and further research is needed to identify and characterize this allergen, and to assess its *in vivo* importance. Oral challenges with Ara h1, Ara h2, Ara h3 and other potentially IgE-reactive allergens are ultimately needed to confirm the true *in vivo* relevance of the individual peanut allergens.

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