

Comparative potency of Ara h 1 and Ara h 2 in immunochemical and functional assays of allergenicity

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Abstract

To assess the relative potency of the major peanut allergens, Ara h 1 and Ara h 2, we examined the relative ability of purified proteins to bind IgE on immunoblots, to cross-link allergen specific IgE in an in vitro assay of degranulation based on RBL SX-38 cells, and to bind IgE in the ImmunoCap[®] assay. Sera from 12 highly sensitive, peanut allergic patients were studied in all assays. IgE immunoblots with crude peanut extracts showed binding of IgE to multiple bands including the 63 kDa and 17–19 kDa bands that contain Ara h 1 and Ara h 2, respectively. In the functional assay, Ara h 2 was more potent than Ara h 1 in 11 of 12 sera tested with a median potency that was 52.5-fold more than Ara h 1 ($P < 0.005$). Contrary to findings with the functional assay, IgE immunoblots with purified Ara h 1 and Ara h 2 showed substantially lighter binding of IgE to Ara h 2 compared with Ara h 1 ($P = 0.02$). The ImmunoCap[®] assay gave intermediate results with slightly more IgE binding to Ara h 2 than to Ara h 1 ($P = 0.005$). In conclusion, Ara h 2 is a very potent allergen and is much more potent than Ara h 1 for most sera using an in vitro assay of IgE cross-linking and cell activation. This finding is different from what was predicted based on immunoblots or with the ImmunoCap[®] assay.

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Introduction

Hypersensitivity to foods is now recognized as a worldwide problem in the industrialized world [1]. In the United States, there are an estimated 4 million Americans with well-substantiated food allergies [2]. In Montreal, 1.5% of early elementary school students were found to be sensitized to peanuts [3]. Reactions to foods are thought to be the most common cause of anaphylaxis when it occurs outside of the

hospital and are estimated to cause 125 deaths per year in the US [4]. An analysis of 32 fatalities thought to be due to food-induced anaphylaxis revealed that peanut was the likely responsible food in 62% of the cases [5]. In placebo-controlled food challenges, peanut-sensitive patients can react to as little as 100 µg of peanut protein [6]. Thus, anaphylactic and other allergic reactions to peanuts are significant medical problems [1,4,7]. Treatment with anti-IgE increases the threshold dose of peanut that causes a clinical reaction but does not cure the disease [8]. Immunization with engineered, recombinant peanut proteins and with soy proteins have shown promise in murine models of peanut allergy [9–11].

The peanut, *Arachis hypogaea*, is a member of the legume family and is related to beans and peas, but not to tree nuts [12,13]. Immediate hypersensitivity reactions to peanuts

Abbreviations: CPE, crude peanut extract; FcεRI, the high affinity receptor for IgE; RBL SX-38 cells, Rat Basophilic Leukemia (RBL 2H3) cells expressing the α, β, and γ chains of human FcεRI.

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are unusual in that they are much more prevalent and severe than for other legumes and tend to persist into adulthood [2]. For these reasons, substantial effort has been made to identify the major peanut allergens in hopes of desensitization of patients to the important peanut proteins or production of peanuts without allergenic activity [14–16]. Nine potentially important allergens of peanut have been identified (Ara h 1–Ara h 8 plus peanut oleosin) [17–22]. Ara h 1 (63 kDa) and Ara h 2 (17–19 kDa doublet) have been designated the major peanut allergens, based on the frequent and intense binding of IgE to these proteins on immunoblots with sera from >90% of peanut-allergic patients [14]. However, in other studies, the percentage of patients whose sera bind to Ara h 1 and Ara h 2 ranges from 40 to 90% [19,20,23,24]. We have previously reported in abstract form that Ara h 2 is a more potent allergen than is Ara h 1 in an in vitro model of allergen function [25]. Confirmatory data using basophil histamine release (BHR) and percutaneous skin tests have been reported by Koppelman et al. with further extension that Ara h 2, in addition to being more potent than Ara h 1, is also more potent than Ara h 3 [26].

RBL SX-38 cells are rat basophilic leukemia cells that stably express approximately 100,000 copies per cell of the α , β and γ chains of the human high affinity receptor for IgE, Fc ϵ RI [27]. This receptor gives 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 [27,28]. We assessed quantitatively the ability of the major peanut allergens, Ara h 1 and Ara h 2, to degranulate RBL SX-38 cells following sensitization of the cells with IgE

from each of 12 subjects with documented sensitivity to peanuts and compared these functional results with findings on IgE immunoblots and with the ImmunoCap[®] assay.

Methods

Patients, sera and IgE levels

This study was approved by the Institutional Review Boards of the University of Colorado Health Sciences Center, the National Jewish Medical and Research Center, and the University of Arkansas School of Medicine. All subjects or their guardians signed informed consent. Sera were evaluated from 31 patients with a history of severe reactions to peanuts. Sera from the 13 patients who were not studied further had relatively low amounts of anti-peanut IgE (<0.35 to 9.7 kU/l) and either did not sensitize the RBL SX-38 cells sufficiently for degranulation to CPE or did not show any bands on immunoblot with purified Ara h 1 and Ara h 2. Eighteen (58%) of these were found to have sufficiently high levels of anti-peanut IgE to be active in our in vitro assay (see reference [28]). Sufficient serum was available from 12 of these patients to complete all of the studies reported here (Table 1). The other 6 sera were assessed in at least one of these assays and gave similar findings (data not shown). These 12 subjects ranged in age from 5 to 50 years old at the time the sera were collected. The sera were collected months to years after the last known exposure to peanuts. Anti-peanut IgE levels ranged from 13

Table 1
Symptoms, anti-peanut IgE, and allergen-specific IgE

Subject	Symptoms	IgE (kU/l)	Anti-Peanut IgE (kU/l)	% of total IgE	Anti-Ara h 1 IgE (kU/l)	Anti-Ara h 1 IgE (% of anti-peanut)	Anti-Ara h 2 IgE (kU/l)	Anti-Ara h 2 IgE (% of anti-peanut)	Anti-Ara h 1 plus Anti-Ara h 2 IgE (% of anti-peanut)
B8	Hives/AE	298	142	48	122	86	26	18	104
B10	Hives/Resp./GI	639	13	2	<0.35*	3	6.5	50	53
B20	Hives/AE	871	533	61	249	47	184	35	81
B119	Hives/Resp.	324	120	37	14	12	92	77	88
D10	Syst. Anaphyl.	52	20	38	4.1	21	7.9	40	60
D13	Hives/AE	1465	17	1	<0.35*	2	12.8	75	77
D17	Resp./AE	180	44	24	5.5	13	10.7	24	37
D18	Hives/Resp.	161	64	40	19	30	15	23	53
D19	Resp./Hives	124	48	39	8.4	18	23	48	65
D26	Syst. Anaphyl.	343	90	26	48	53	36	40	93
D29	Resp./AE	106	26	25	4.4	17	15	58	75
E2	Respiratory	224	102	46	17	17	47	46	63
Mean SD				32 $\pm$ 18		26 $\pm$ 24		44 $\pm$ 19	71 $\pm$ 19
Geometric mean		246	57		10		23		
95% confidence intervals		140–462	29–113		3–35		12–39		
P value ^a							ns, P = 0.06	ns, P = 0.06	

Abbreviations for this table: Resp. = respiratory; AE, =angioedema; Syst. Anaphyl. = systemic anaphylaxis.

^a Paired *t* tests using log values comparing IgE anti-Ara h 2 to anti-Ara h 1 as raw values (U/l) and as % of total anti-peanut IgE.

* For values <0.35 U/l, 0.35 was used for calculations.

kU/l to 533 kU/l. Eleven had anti-peanut IgE >14 kU/l, a level shown to predict a positive double-blind oral peanut challenge with a positive predictive value of 95% [29]. The remaining serum contained 13 kU/l of anti-peanut IgE and was from a patient with an excellent history of peanut-induced systemic reactions. Sera were stored in aliquots at -20°C . All 12 sera gave detectable binding of IgE on immunoblots and were able to sensitize the RBL SX-38 cells for degranulation in the functional assay. Four of the 12 sera studied were initially found to be toxic to the RBL SX-38 cells causing cell death and/or high background release of serotonin. The toxicity appears to be due to naturally occurring xenoreactive antibodies [30]. These problems were overcome by removal of IgG from the toxic sera under conditions that do not remove IgE and by adding a preincubation step followed by an additional wash step immediately prior to triggering the cells (see below).

Reagents

The myeloma IgE, goat anti-IgE, and other reagents were as previously described [27,28]. The crude peanut extract (CPE) was a standard skin test extract stored in 50% glycerol at 4°C and obtained from Alk Abello (Round Rock, TX).

Purified Ara h 1 and Ara h 2, and estimation of potency

Ara h 1 and Ara h 2 were purified from a crude peanut extract using anion and cation exchange chromatography as described [31,32]. These purified proteins were stored in aliquots at -20°C and diluted prior to each experiment. On gels, Ara h 1 runs at 63 kDa and Ara h 2 runs as a doublet with an apparent molecular weight of 17–19 kDa. The Ara h 1 contained a minor breakdown product and/or contaminant (~45 kDa) accounting for less than 5% of the material (data not shown). There has been no increase in the amount of the smaller molecular weight material over a 3-year period. Each purified protein was run on a Superdex 200 column (Amersham Biosciences, Piscataway, NJ) and no aggregates were seen. We estimated potency by determining the concentration of Ara h 1 and Ara h 2 that gave 50% of the degranulation seen with an optimal dose of CPE ($1:10^5$ dilution) for each serum (see below). This is defined as an EC₅₀ as described by MacGlashan et al. [33,34]. For comparison of EC₅₀ between these allergens, pmolar (pM) values rather than weight per volume (ng/ml) were used.

IgE immunoblots

IgE immunoblots were performed with CPE and with purified Ara h 1 and Ara h 2. In the first set of experiments, 10–20 μg of CPE was run on a 4–20% polyacrylamide gel and transferred to nitrocellulose. For 5 sera, immunoblots were developed with ^{125}I anti-human IgE reagents as previously described [32]. For the remaining 7 sera, the

immunoblots were blocked for 2 h with 3% milk powder in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), probed overnight at 4°C with dilutions of sera (1:5–1:20, yielding 5–27 ng/ml of anti-peanut IgE), and developed with horseradish peroxidase (HRP)-conjugated mouse anti-human IgE (Zymed, San Francisco, CA) followed by an enhanced chemiluminescence reagent, Luminol® (Santa Cruz Biotech, Santa Cruz, CA) for 1–30 min. Both of these methods were performed with 4 sera and gave equivalent results. In the second set of experiments, 125 pmol of Ara h 1 (8 μg) and 125 pmol of Ara h 2 (2 μg) were applied to the first two lanes and 470 pmol of Ara h 2 (8 μg) were applied to the third lane. After transfer, the nitrocellulose was processed and developed as described above. To estimate the relative binding on immunoblot, densitometry measurements for IgE binding to Ara h 2 were divided by data for Ara h 1. If an Ara h 2 band was only visible at the higher concentration, the density was adjusted by the molar difference of the proteins in the respective lanes.

ImmunoCap® assay with purified allergens

Purified Ara h 1 and Ara h 2 were incorporated into the ImmunoCap® format by Pharmacia (Upsala, Sweden) and were analyzed in the Clinical Laboratory of the National Jewish Medical and Research Center with appropriate controls as specified by the manufacturer.

Maintenance of RBL SX38 cells in tissue culture

RBL SX-38 cells are RBL 2H3 cells that have been stably transformed to express the α , β and γ chains of human Fc ϵ RI [27]. The cells were grown in Eagle's Minimal Essential Medium (Gibco) culture medium (CM) with 10% fetal calf serum (HyClone), 1% penicillin/streptomycin (Gibco) and 1.2 mg/ml G418 (Geneticin, Invitrogen) to maintain expression of the transgene as previously described [27]. Expression of human Fc ϵ RI was monitored by flow cytometry with myeloma IgE (IgEps) followed by biotin-anti-IgE and FITC-avidin as previously described [28].

Preparation of RBL SX38 cells for assay

This assay has been previously described [28]. Briefly, 2 days prior to a planned experiment, cells were plated at $\sim 2 \times 10^5$ cells/ml (200 μl /well) with 1 μCi /ml tritiated 5-hydroxytryptamine (5-HT, serotonin) but without G418. One day prior to an experiment, serum (from an allergic donor) was diluted 1:5 to 1:20 (depending upon the concentration of IgE) in EMEM without fetal calf serum. Sera were either cross-absorbed for 1 h at 37°C against RBL 2H3 cells (lacking human Fc ϵ RI) to specifically remove xenoreactive antibodies or incubated with protein G agarose to remove all IgG [35]. The treatment with protein G was more effective and is now our standard procedure (data not shown). The treated serum was then added to the RBL SX-38

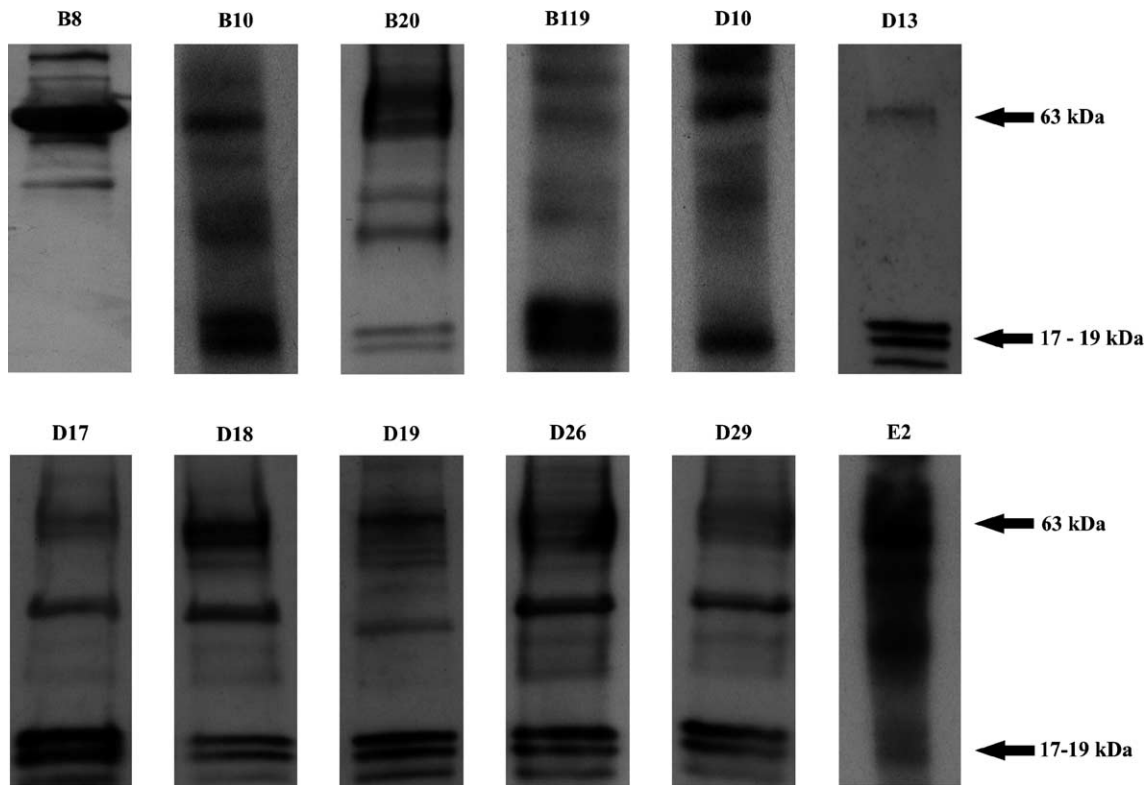


Fig. 1. IgE immunoblots of crude peanut extract with sera from peanut-sensitive patients. Immunoblots were performed as described in Methods, giving similar results. Sera were diluted 1:5 to 1:33 to give a final concentration of anti-peanut IgE of 5 to 27 ng/ml.

cells (washed twice with PBS) for 24 h. For each experiment, cells were washed three times with a NaPIPES buffer and then in the same buffer with 100 μ M NECA (5'-N-ethyl-carboxamide) as previously described [28]. For some, sera background was reduced by adding an extra 30 min incubation in the NaPIPES buffer and then three more washes before adding the buffer containing NECA and triggering.

RBL SX38 cell triggering by specific allergens

The labeled and sensitized RBL SX-38 cells were triggered with anti-IgE (3 μ g/ml), crude peanut extract (1:100,000 dilution; 150 ng/ml), purified Ara h 1, or purified Ara h 2 at the concentrations noted (0.25–25,000 ng/ml). Percent release was calculated as previously

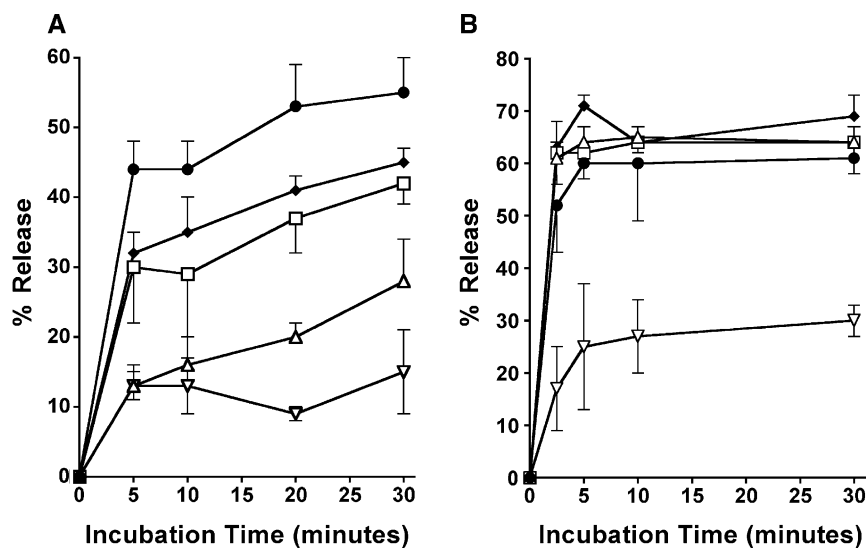


Fig. 2. Time course of degranulation for RBL SX-38 cells sensitized with sera from peanut-allergic donors. Cells were triggered with anti-IgE (3 μ g/ml; closed circles), CPE (1:100,000 dilution = 150 ng/ml; closed diamonds), Ara h 2 (250 ng/ml = 15 nM; open squares), Ara h 1 (2500 ng/ml = 39 nM; open triangles), or buffer (open inverted triangles) for the times shown. (A) subject D17; (B) subject B20. Error bars are one SD for triplicate observations.

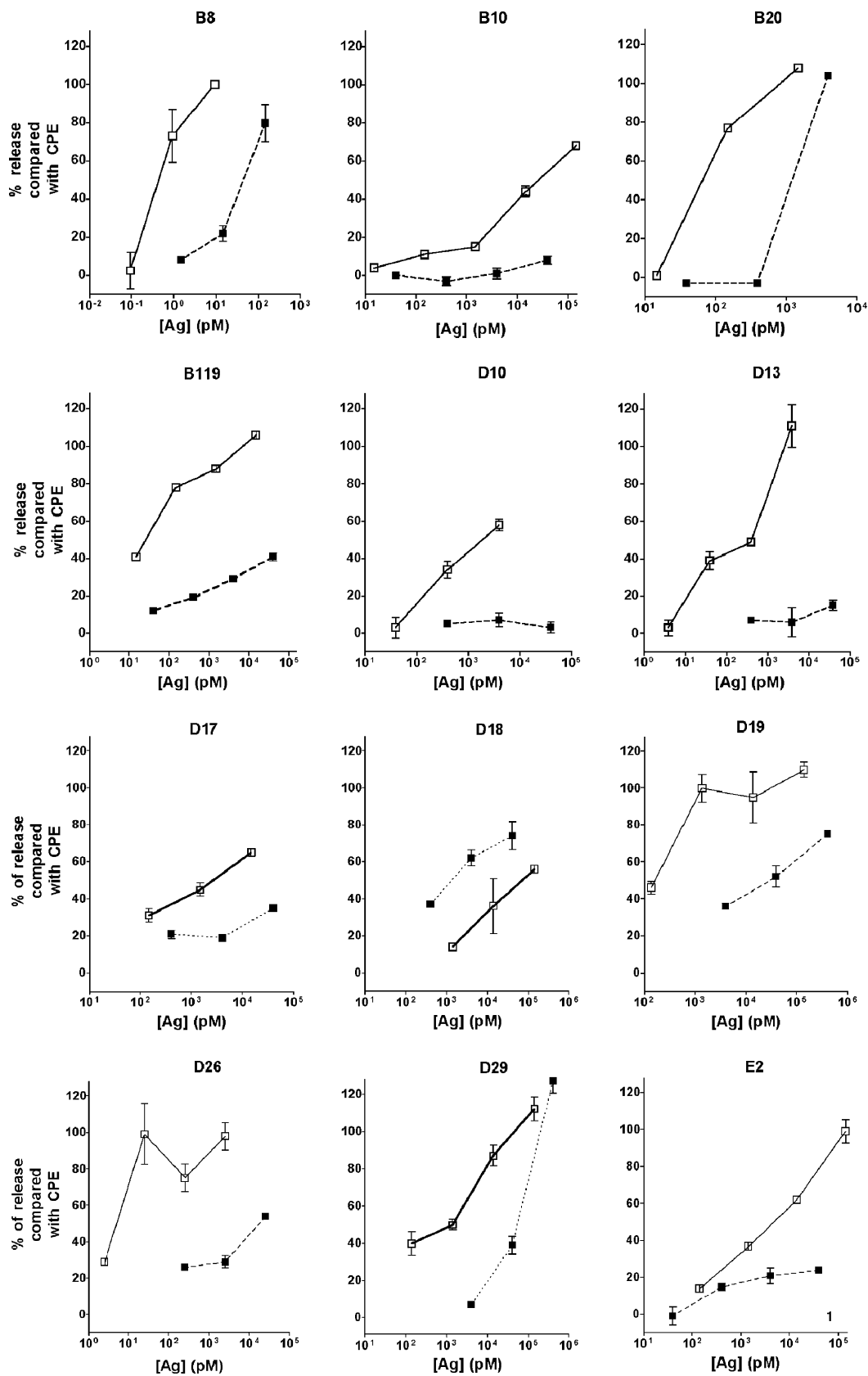


Table 2
EC50 values for Ara h 1 and Ara h 2

Subject	EC 50 Ara h 1 (ng/ml)	EC 50 Ara h 2 (ng/ml)	EC 50 Ara h 1 (pM)	EC 50 Ara h 2 (pM)
B8	147	15	2300	830
B10	≥64,000	510	≥1,000,000 ^a	28,000
B20	63	1.4	1000	78
B119	>2500	2.1	>40,000 ^a	120
D10	≥320	206	≥20,000 ^a	3500
D13	7000	17	111,000	940
D17	2600	10	41,000	560
D18	230	2000	3700	111,000
D19	21,200	32	336,000	1800
D26	1600	0.6	25,000	33
D29	7600	43	121,000	2400
E2	≥10,000	49	≥160,000 ^a	2900
Geometric mean (minimal value)	≥1700	19	≥29,600	887
Upper 95% confidence interval	7100	100	128,000	4688
Lower 95% confidence interval	470	5	8241	233
<i>P</i> value ^b		0.003		0.001

^a Minimal EC50 values for experiments where the highest doses of Ara h 1 tested did not achieve 50% of the value with CPE were determined by extrapolating the results with the highest dose tested along a line that parallels the slope seen with Ara h 2. Molar values were calculated based on molecular weights of 63 kDa for Ara h 1 and 18 kDa for Ara h 2.

^b Paired *t* test using log values comparing Ara h 1 with Ara h 2.

described [27,28]. In these experiments, the background degranulation was $25 \pm 4\%$ (mean $\pm$ SEM; $n = 12$). With anti-IgE, the total degranulation was $61 \pm 3\%$ (mean $\pm$ SEM; $n = 12$) and with CPE it was $59 \pm 5\%$ (mean $\pm$ SEM; $n = 12$). After subtracting the background for each experiment, the net release was $36 \pm 3\%$ with anti-IgE (mean $\pm$ SEM; $n = 12$) and $35 \pm 5\%$ with CPE (mean $\pm$ SEM; $n = 12$). The signal with anti-IgE and with CPE was always 1.5–4 times background. To make comparisons among experiments, release following exposure to the allergens can be expressed as a percentage of that seen with optimal doses of either anti-IgE or CPE in the same assay. In this report, release with optimal amounts of CPE was used as the standard instead of anti-IgE because the maximum level of degranulation that any single allergen may achieve could equal but should not exceed the level seen with the whole extract. If data were recalculated using anti-IgE as the reference, the absolute values would decrease slightly since, for these sera, optimal doses of CPE gives about 80–100% of the level of degranulation seen with anti-IgE. The comparative data would not change.

Statistical analysis

Statistical comparisons were all two-tailed. *P* values < 0.05 were considered to be statistically significant. Spearman correlation coefficients were calculated except for the

immunoglobulin levels where geometric data generated a Gaussian distribution and Pearson correlations were used. Otherwise, the specific tests used are specified in the figure and table legends. All analysis was generated with GraphPad Prism version 4.0 b software for the Macintosh (GraphPad Software, San Diego CA).

Results

Immunocap® assays with unpurified and purified allergens

A substantial proportion of the total IgE (geometric mean = 246 kU/l) could be accounted for by binding to unpurified peanut proteins (geometric mean = 57 kU/l) (Table 1). Similarly, much of the IgE binding to unpurified peanut proteins could be accounted for by binding to purified Ara h 1 (geometric mean = 10 kU/l) and/or Ara h 2 (geometric mean = 23 kU/l). Based on the ImmunoCap® assay, $32 \pm 18\%$ of the total IgE is anti-peanut and, of the anti-peanut IgE, $26 \pm 24\%$ is anti-Ara h 1 and $44 \pm 19\%$ is anti-Ara h 2. Overall, $79 \pm 19\%$ of the anti-peanut IgE can be accounted for by binding to either Ara h 1 or Ara h 2. The amount of IgE binding to Ara h 1 and to Ara h 2 correlates with the amount of anti-peanut IgE (Pearson $r = 0.95$, $P < 0.001$ and Pearson $r = 0.94$, $P < 0.001$, respectively) and correlate with each other (Pearson $r = 0.81$, $P = 0.001$).

Fig. 3. Functional assay of IgE cross-linking. RBL SX-38 cells were sensitized with sera and were stimulated with purified Ara h 1 (closed squares), Ara h 2 (open squares), buffer (not shown), and CPE (150 ng/ml, not shown). Within each experiment, spontaneous degranulation was subtracted from total degranulation with CPE to give the “net degranulation” as described in Methods. The activity with purified Ara h 1 and Ara h 2 are expressed as a percentage of the “net degranulation” seen with CPE. These data are representative of one to three separate functional assays for each serum. Error bars are one standard deviation for triplicate observations. If error bars are not visible, they are smaller than the symbol. The concentrations of Ara h 1 and Ara h 2 shown on the horizontal axis varied among the assays due to variable sensitivity to the allergens for these sera.

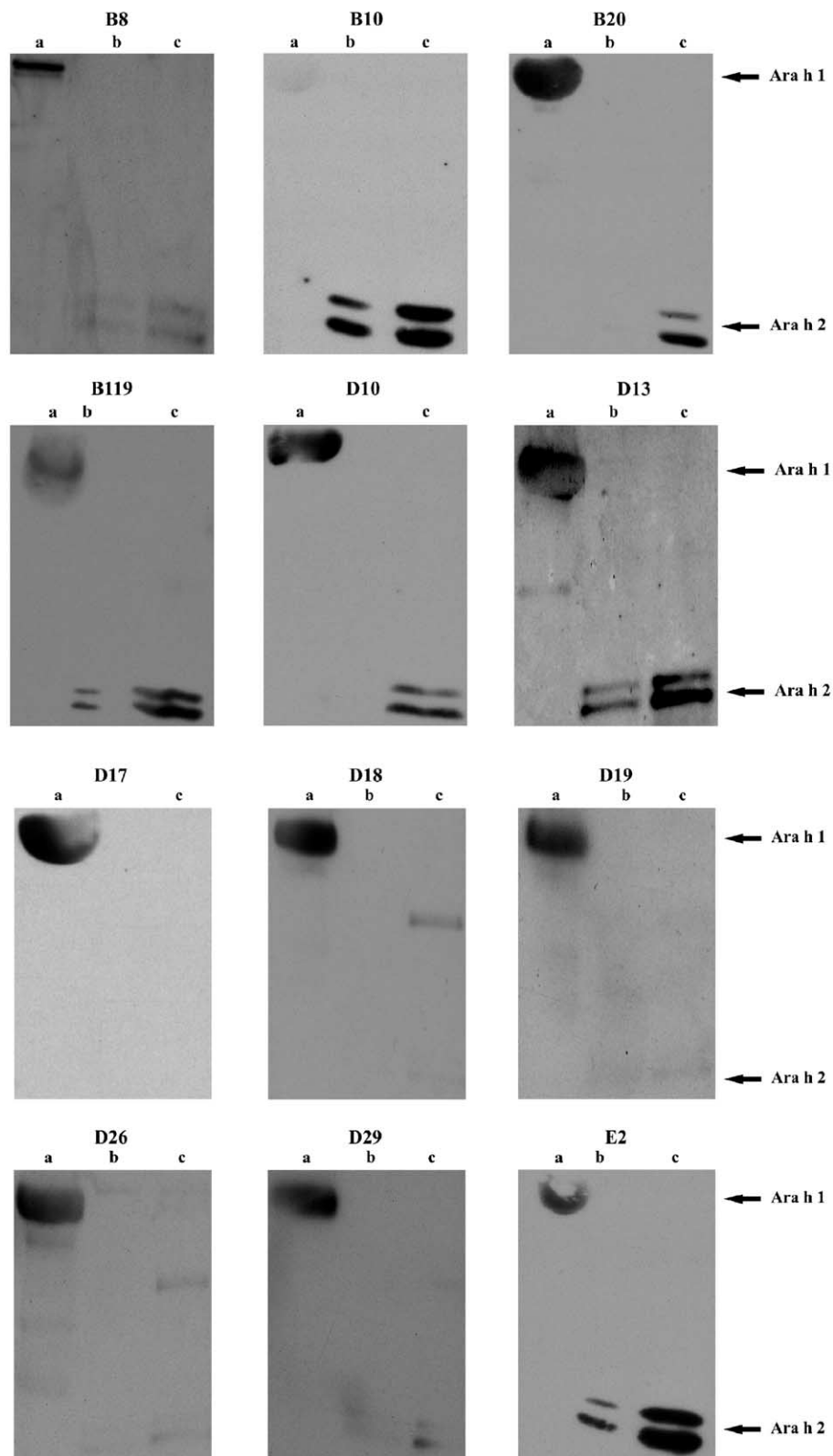


Fig. 4. IgE immunoblots of purified Ara h 1 and purified Ara h 2. 125 pmol (8 μ g) of Ara h 1 was loaded in lane a, 125 pmol (2 μ g) of Ara h 2 was loaded in lanes b, and 470 pmol (8 μ g) of Ara h 2 was loaded in lane c. The blot for serum D10 only contained lanes a and c. Sera were diluted 1:5 to 1:33 to give a final concentration of anti-peanut IgE of 5 to 27 ng/ml as in Fig. 1. These data are representative of one to three separate immunoblots for each serum. Sera D17 and D18 showed no binding to Ara h 2. An artifact at $\sim$ 40 kDa is visible in lane c of blots with sera D18 and D26.

Immunoblots with crude peanut extracts

In several previously published studies, the frequency of peanut-sensitive patients whose IgE recognized Ara h 1 and Ara h 2 was determined by immunoblotting crude peanut extracts (CPE) with patient sera and examining binding of IgE to bands at 63 kDa and 17–19 kDa, respectively [14,32]. In order to compare the sera studied here with those in previous reports, immunoblots using CPE were performed (Fig. 1). The 63-kDa band and 17–19 kDa doublet that correspond in MW to where Ara h 1 and Ara h 2 are found are noted with arrows. As previously reported, the IgE in most of these sera identify multiple bands including the prominent bands at 63 kDa (all 12 sera) and 17–19 kDa (11 of 12 sera).

Functional assay with RBL SX-38 cells: time course of secretion

To characterize the ability of purified Ara h 1 and Ara h 2 to degranulate RBL SX-38 cells that were sensitized with IgE from peanut allergic patients, time course experiments were performed with anti-IgE, CPE, purified Ara h 1, and purified Ara h 2. Based on preliminary assays (not shown), the concentrations of each reagent were chosen to give maximal signals for the sera shown. For serum D17 (Fig. 2A), CPE and Ara h 2 gave a time course and level of degranulation similar to that seen with anti-IgE. Ara h 1 was less effective with this serum. For serum B20 (Fig. 2B), optimal doses of anti-IgE, CPE, Ara h 1 and Ara h 2 all caused rapid degranulation with similar maximal signals. The 20-min time point was chosen for subsequent studies. In control experiments, RBL SX-38 cells sensitized with myeloma IgE (PS) or with IgE from a non-peanut-sensitive subject responded to anti-IgE but not to CPE, Ara h 1, or Ara h 2 (data not shown).

Functional assay with RBL SX-38 cells: comparison of Ara h 1 and Ara h 2 with calculation of EC50

The ability of Ara h 1 and Ara h 2 to cross-link IgE from these sera was assessed with the RBL SX-38 cells. Individual assays are shown in Fig. 3 and the EC50 data are compiled in Table 2. For 11 of 12 sera, Ara h 2 is more potent than is Ara h 1. The exception is serum D18, for which Ara h 1 is more potent (Fig. 3). For 4 of the 12 sera, the EC50 of Ara h 1 had to be derived by extrapolation since there was minimal or no activity detected at the highest concentration tested (40,000 pM or 2500 ng/ml for earlier experiments and 400,000 pM or 25,000 ng/ml for later experiments). The functional assays show that Ara h 2 is very active with an EC50 of 19 ng/ml (geometric mean), whereas Ara h 1 is much less active with an EC50 of ≥ 1700 ng/ml (geometric mean) ($P = 0.003$, $n = 12$) (Table 2). These data were then recalculated based on pmol of allergen rather than ng. This analysis also shows that Ara h 2 is more potent with an EC50 of 887 pmol/l compared

to an EC50 for Ara h 1 of $\geq 29,600$ pmol/l ($P = 0.001$). For these sera, there was no significant correlation between the EC50 for Ara h 1 and for Ara h 2.

Immunoblots with purified Ara h 1 and Ara h 2

Since the functional data showed that Ara h 2 was more potent than Ara h 1 and the immunoblots with CPE showed variability in binding to the 63-kDa and the 17- to 19-kDa regions (not quantified), immunoblots were performed with purified Ara h 1 and Ara h 2. The immunoblots show that purified Ara h 1 was recognized by IgE from all 12 sera and the bands were darker for Ara h 1 than for Ara h 2 with 11 of 12 sera (Fig. 4 and Table 3). On the other hand, only 8 of the 12 serum samples contained IgE that gave visible bands with purified Ara h 2, even at 4-fold higher molar amounts (Fig. 4).

Comparison of functional activity of Ara h 1 and Ara h 2 with binding of IgE on immunoblots and with ImmunoCaps®

Summary data from Table 1 (ImmunoCap® assay), Table 2 (functional assay) and from densitometric analysis of photographs of IgE binding to purified Ara h 1 and Ara h 2 (Fig. 4) are shown from left to right in Table 3. In the ImmunoCap® assay, Ara h 2 binds more IgE than does Ara h 1 (median ratio

Table 3
Relative activities of Ara h 1 and Ara h 2

Subject	Relative binding in ImmunoCap® Assay Ara h 2: Ara h 1 ^a	Relative potency with RBL SX-38 cells Ara h 2: Ara h 1 ^b	Relative binding on Immunoblot Ara h 2: Ara h 1 ^c
B8	0.21	2.8	0.66
B10	>19	≥ 36	311
B20	0.74	13	0
B119	6.6	>342	0
D10	1.9	≥ 5.7	0.11
D13	>37	117	0
D17	1.9	74	0.28
D18	0.79	0.033	0.01
D19	2.7	189	0
D26	0.75	769	0.04
D29	3.3	50.4	0.16
E2	2.8	≥ 55	0.11
Median	2.3	52.5	0.07
P value	$P = 0.005^d$	$P < 0.005^d$	$P = 0.02^d$

Friedman's two-way analysis by ranks was performed followed by Dunn's multiple comparison test. The relative potency for Ara h 2 vs. Ara h 1 in the functional assay was significantly different from the relative binding on immunoblot ($P < 0.001$). The other comparisons were not significantly different.

^a Ratios of data shown in Table 1.

^b Ratios of data shown in Table 2. The relative potency of Ara h 2 compared with Ara h 1 is the ratio of the EC50 (in pM) for Ara h 1 divided by the EC50 for Ara h 2.

^c Ratios of intensities on immunoblots are from Fig. 4.

^d Wilcoxon signed rank test comparing each of the observed median values to a hypothetical value of 1.0. For statistical comparisons, values shown as > or $\geq$ were analyzed as the minimal value.

of binding of IgE to Ara h 2 compared with Ara h 1 = 2.3; different from equal binding, $P = 0.005$). In the functional assay, Ara h 2 is active at lower concentrations and is therefore more potent (median ratio of EC50 for Ara h 1 compared with Ara h 2 = 52.5; different from equal potency, $P < 0.005$). In the immunoblot assay with purified allergens, Ara h 2 binds less IgE (median ratio of 0.07; different from equal binding, $P = 0.02$). Further analysis using Friedman's two-way analysis by ranks to compensate for multiple comparisons revealed that the relative potency of Ara h 2 compared with Ara h 1 in the functional assay was significantly different from the relative binding on immunoblot with purified allergens ($P < 0.001$), whereas the other comparisons were not significantly different.

Eight of the 12 sera bound Ara h 2 predominantly in the ImmunoCap® assay and for 11 of the 12 sera, Ara h 2 was more potent in the functional assay (Tables 1, 2, and 3). This is marked contrast to the finding that IgE from 11 of 12 of these sera bound Ara h 1 with a more intense signal in the immunoblot assay. Correlations of the data among these assays were as follows: ImmunoCap® and functional assay, Spearman $r = 0.40$ ($P = 0.20$; ns); ImmunoCap® and immunoblots, Spearman $r = -0.08$ ($P = \text{ns}$); and immunoblot and functional assay, Spearman $r = -0.39$, ($P = 0.22$, ns).

Discussion

Binding of IgE from highly peanut-sensitive patients to purified Ara h 1 and Ara h 2 was assessed by IgE immunoblots, binding of IgE in the ImmunoCap® assay with purified Ara h 1 and Ara h 2, and by the ability of these purified proteins to function by activating sensitized RBL SX-38 cells. Although these assays display the allergens of interest in different ways (e.g., ImmunoCap®: solid phase, not denatured; immunoblot, solid phase, denatured; functional assay: soluble, native) and likely have different dominant epitopes, each has the capacity to assess differences in binding of IgE to purified and/or recombinant allergens. IgE from individual patients may recognize different epitopes and thus have variable activity in each assay. Others have assessed allergenicity of antigens with immunoblotting, RAST inhibition, BHR and skin testing [22,26,36–39].

In that a quantitative comparison of immunochemical and functional assays for peanut allergens has not been previously published, we chose to measure the relative abilities of purified Ara h 1 and Ara h 2 to bind IgE on immunoblots, in the ImmunoCap® assay, and in a functional assay. Since there is no reference standard for binding of IgE on immunoblots, we examined the relative binding of Ara h 2 compared with Ara h 1 and report this as fold difference. We further used the functional assay to calculate EC50s for each allergen.

The first set of immunoblots examined the binding of IgE to crude peanut extracts (CPE) (Fig. 1). Findings with the sera

used in this assay are consistent with the previously reported prominence of IgE binding to both 63 kDa and 17–19 kDa bands that contain Ara h 1 and Ara h 2, respectively [14]. Most sera identify other bands as well. Next, purified Ara h 1 and Ara h 2 were incorporated into the ImmunoCap® assay and binding of IgE to each was measured. Binding of IgE to these purified proteins accounts for $79 \pm 19\%$ of the total anti-peanut IgE in these sera. Overall, Ara h 2 bound more IgE from these sera in the ImmunoCap® assay than did Ara h 1 ($P = 0.02$) (Tables 2 and 3). We then performed functional assays with purified Ara h 1 and Ara h 2 and found that Ara h 2 was markedly more potent than Ara h 1 for 11 of 12 sera (Fig. 3). Koppelman et al. recently published data that support our observations (presented first as an abstract and now in detail) that Ara h 2 is functionally more potent than is Ara h 1 [25,26]. Finally, immunoblots with purified allergens were then examined and revealed an unexpected pattern with the intensity of the Ara h 2 bands being much less than that of the Ara h 1 bands (median difference of 0.07, $P = 0.02$, Table 3 and Fig. 4). It is important to note that the assessment of the intensity of the bands in the immunoblots is at best, semi-quantitative and is only meant to convey the relative intensity of the bands. These blots are not indicators of affinity. Similar discrepancy between binding of proteins on immunoblot and clinically important activity has been reported with Der p 1 [40].

Ara h 2 was active at lower concentrations for 11 of 12 sera in the functional assay yet binding of IgE from these sera to Ara h 1 in the immunoblot assay was more intense than the binding of IgE to Ara h 2 (Table 3, $P < 0.001$). However, there was not a statistically demonstrable correlation between the relative potency of Ara h 2 compared with Ara h 1 in the functional assay and the levels of anti-peanut IgE from the ImmunoCap® assay (data from Tables 1 and 3). This may be due to the relatively small sample size. A review of the clinical histories for these patients did not reveal any distinguishing characteristics that might correlate with the observed immunoreactivity.

RBL SX-38 cells form the basis of a very sensitive functional assay that is able to detect pg amounts of allergen [28]. These cells have the disadvantage that only sera with high concentrations of allergen-specific IgE and relatively high percent of anti-peanut IgE can be effectively studied. However, given sera with anti-peanut IgE >10 kU/l and anti-peanut IgE that is $>5\%$ of the total IgE, this assay has several advantages. First, these cells contain only empty receptors and can easily be sensitized with sera from multiple allergic donors without the need for frequent phlebotomy or stripping away IgE (as in the BHR assay). Secondly, these cells are dependably responsive, whereas a small number of donors (up to 10%) have non-responsive basophils. Finally, experiments do not depend upon the frequent availability of specific peanut-allergic donors. A single sample of serum can be used for multiple assays over a long period of time. Although this is a model system, the important part of the biological response, the interaction between allergen-specific IgE and

allergen, is entirely human. The data in Fig. 3 and Table 2 demonstrate that, given sera with high amounts of anti-peanut IgE, this assay can detect very small amounts of allergen and can be used to establish EC50 values.

The EC50s for Ara h 2 varied from 0.6 to 2000 ng/ml, whereas the CPE gave maximal activity at 150 ng/ml. This suggests that either there are other important proteins in the CPE other than Ara h 2 for a substantial number of sera or that our purified Ara h 2 was not of full potency. So it is an important assumption that the purified Ara h 1 and Ara h 2 have the same or nearly the same relative specific activity (ability to cross-link IgE) that they had prior to purification. Although this has not been proven, this is likely the case for the following reasons: (1) these proteins were purified by salt exchange columns and not by a denaturing process. (2) Both Ara h 1 and Ara h 2 have the correct appearance on gels and are known to be quite stable. We have not seen decay in the chromatographic profile or in the functional activity of either Ara h 1 or Ara h 2 over >12 months of storage at -20°C . Furthermore, Ara h 1 and Ara h 2 are reported to be extraordinarily stable, surviving both food-processing methods and in vitro digestion systems [41–43]. (3) Ara h 2 is very potent with EC50s of <20 ng/ml for 6 of the 12 sera (Fig. 3 and Table 2). For comparison, the model allergen, DNP25BSA at 10 ng/ml, maximally activates RBL 2H3 cells sensitized with monoclonal anti-DNP IgE [44] and has an EC50 compared with anti-IgE of ~ 0.3 ng/ml (data not shown). (4) We obtain the same rank order of potency between Ara h 1 and Ara h 2 reported independently by Koppelman using two different assays, BHR and in vivo skin testing [26].

It is possible that purified Ara h 1 and Ara h 2 contain trace amounts of each other or of another active protein. However, this would tend to decrease the EC50 of our Ara h 1 preparation (since Ara h 2 is more active) and if Ara h 2 is contaminated by the less potent Ara h 1, this would increase the EC50 of Ara h 2. Thus, the discrepancy between these two proteins would be underestimated if there were cross-contamination.

Ara h 1 may fail to activate RBL SX-38 cells sensitized with some sera even though most of these sera contain IgE that recognizes Ara h 1 on immunoblot because (1) higher doses are needed or (2) the important IgE binding epitopes may be internal and not exposed when Ara h 1 is in its native, trimeric, state. These epitopes could thus be exposed on the immunoblots and could be clinically relevant following digestion. So, these studies do not exclude an important role for Ara h 1 following digestion.

We considered the possibility that aggregates of Ara h 1 and/or Ara h 2 are present in solution but not on gels where the proteins are reduced and denatured. This could explain the discrepancy between the functional assay and the immunoblots and the differences in potency between Ara h 1 and Ara h 2. The same preparations of Ara h 1 and Ara h 2 used in these studies were analyzed on a Superdex 200 gel filtration column, aggregates accounted for <1% of the total protein.

In summary, we have examined the ability of IgE from highly sensitive patients to bind to Ara h 1 and Ara h 2 in the ImmunoCap[®] assay, in a functional assay of IgE cross-linkage on RBL SX-38 cells, and on immunoblots. The data presented demonstrate that Ara h 2 is a very potent allergen, activating sensitized cells at very low concentrations and, for most of the sera studied, is a much more potent allergen than is Ara h 1. Examination of relative binding of IgE from serum of highly allergic peanut sensitive donors to 17–19 kDa and 63 kDa proteins in crude peanut extracts, to purified Ara h 1 and Ara h 2 on immunoblots, or in the ImmunoCap[®] assay with purified Ara h 1 and Ara h 2 leads to different estimates of IgE binding. None of these immunochemical assays predict the relative ability of these allergens to cross-link IgE in Fc ϵ RI/IgE complexes and activate the RBL SX-38 cells.

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