

ORIGINAL ARTICLE

Capacity of purified peanut allergens to induce degranulation in a functional *in vitro* assay: Ara h 2 and Ara h 6 are the most efficient elicitors

F. Blanc*, K. Adel-Patient*, M.-F. Drumare*, E. Paty†, J.-M. Wal* and H. Bernard*

*INRA, UR496, Unité d'Immuno-Allergie Alimentaire, CEA-SPI, Gif sur Yvette, Cedex, France and †Université Paris Descartes Assistance Publique des Hôpitaux de Paris, Hôpital Necker Enfants Malades, Paris, Cedex, France

Clinical &
Experimental
Allergy

Summary

Background Peanut is a most common and potent food allergen. Many peanut allergens have been characterized using, in particular, IgE-binding studies.**Objectives** We optimized an *in vitro* functional assay to assess the capacity of peanut allergens to degranulate humanized rat basophilic leukaemia cells, RBL SX-38 cells, after sensitization by serum IgE from peanut-allergic patients. We thus compared the activity of the main peanut allergens, i.e. Ara h 1, Ara h 2, Ara h 3 and Ara h 6, purified from roasted peanut.**Methods** Sera of 12 peanut-allergic patients were collected and total and peanut-specific IgE were measured. They were used to sensitize RBL SX-38 cells and the degranulation was induced by incubation with ranging concentrations of a whole peanut protein extract or of purified peanut allergens. The mediator release was quantified by the determination of β -hexosaminidase activity in the supernatant. The intensity of the degranulation was expressed as maximum release and as EC50, corresponding to the dose of allergen that induced 50% of the maximum release.**Results** For each serum, only 10 IU/mL of human IgE was necessary to sensitize the cells and obtain an optimal degranulation. With all the allergens, the release was positively correlated with the concentration of allergen-specific IgE in the serum used to sensitize the cells. The medians of EC50 obtained for Ara h 2 and Ara h 6 were 2.1 and 2.8 μ M, respectively, while they were much higher for Ara h 3 and Ara h 1 (65 and 150 μ M, respectively).**Conclusion** The RBL SX-38 release assay proved to be sensitive, specific and reproducible. It allowed the comparison of the degranulation potential of different peanut allergens. For all the sera tested, Ara h 2 and Ara h 6 were more potent than Ara h 1 or Ara h 3.**Keywords** IgE, mast cell activation, peanut allergens

Submitted 10 September 2008; revised 9 April 2009; accepted 13 April 2009

Correspondence:

Hervé Bernard, Unité d'Immuno-
Allergie Alimentaire, CEA-SPI, iBiTec-S,
Bat 136, F-91191 Gif sur Yvette cedex,
France.
E-mail: herve.bernard-INRA@cea.fr

Introduction

Peanut allergy is a most common food allergy, with a prevalence of 0.2% in the general paediatric population and 0.4% among adults [1]. Clinical symptoms are usually severe [2] and a majority of fatalities due to food-induced anaphylaxis are caused by peanut [3, 4].

Peanut, *Arachis hypogaea*, belongs to the Leguminosae family and its kernel contains around 25% proteins (w/w). In the past decade, several peanut allergens have been identified. Ninety percent of peanut-allergic patients are sensitized to Ara h 1, which belongs to the 7S globulin protein family [5–7], and 80% are sensitized to Ara h 2, a 2S albumin [5, 8–10]. Recently, Ara h 6, another 2S albumin, was identified as a major peanut allergen [9, 11,

12]. The 11S globulin Ara h 3/4, naturally present in peanut as proteolytically plant-processed polypeptides [13], is recognized by IgE of 35% to 55% of peanut-allergic patients [7, 9, 10, 14].

Most of the studies analysed the IgE-binding capacity of those proteins using *in vitro* immunochemical methods such as enzyme immunoassay or Western blotting. However, these methods do not provide information on the ability of the purified protein to elicit an allergic reaction. For ethical reasons, food challenges and skin testing may not be appropriate for research purposes. Basophil-degranulation tests, which require a processing of blood samples immediately after collection, may also not be suitable. Therefore, an *in vitro* functional test using a humanized stable mast cell line is of considerable interest

to study the biological activity of purified proteins or to study the effect of processing or digestion on their allergenic potential. RBL SX-38 cells are rat mast cells that stably express the α , β and γ chains of the human high-affinity receptor for IgE, Fc ϵ RI [15], allowing IgE of allergic patient sera to bind on their surface. Cells can then be specifically activated by the allergen to which the patient is allergic [15, 16].

The aim of this study was to optimize this cell-based assay and to compare the ability of purified peanut allergens, i.e. Ara h 1, Ara h 2, Ara h 3, Ara h 6 and a glycinin-derived fragment, to activate RBL SX-38 cells following sensitization with IgE of individual sera of patients with documented peanut allergy.

Materials and methods

Equipment and reagents

All enzyme immunoassays were performed in 96-well microtitre plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using specialized Titertek microtitration equipment from Labsystems (Helsinki, Finland). Unless otherwise stated, all reagents were of analytical grade from Sigma (St Louis, MO, USA).

Allergic patients

The studied population included 12 peanut-allergic patients (seven boys and five girls, median age: 10 years)

recruited at the Paediatric Allergy Unit of the Hospital Necker-Enfants Malades (Paris, France) (Table 1). All the patients had confirmed peanut allergy based on an extensive history, physical examination and objective clinical manifestations observed after peanut ingestion. Numerous symptoms were reported involving the skin, respiratory tract, gastrointestinal tract and cardiovascular system. Eight patients presented severe symptoms with anaphylactic shock, angio-oedema or the involvement of three organs. Four patients had mild to moderate reactions involving one or two organs.

Sera of 76 peanut-allergic patients recruited in the same Paediatric Allergy Unit were pooled and used as a positive control. Sera of 10 milk-allergic patients without peanut allergy were also pooled and used as a negative control.

Preparation of whole peanut protein extract and purified allergens

The whole peanut protein extract (WPPE) was prepared from commercial roasted peanuts, and Ara h 1, Ara h 2, Ara h 3, Ara h 6 and glycinin-derived fragment were purified as described previously [11, 13, 17]. The glycinin-derived fragment corresponds to a large peptide naturally generated after the proteolytic plant processing of Ara h 3 in the peanut seed. The molecular weight (MW) of each purified allergen was determined by MALDI-TOF mass spectrometry (Voyager DE RP apparatus from PE Biosystems, Foster City, CA, USA). The values obtained

Table 1. Serological and clinical data for the 12 peanut-allergic patients

Sera	Symptoms	Age	Gender	Total IgE (IU/mL)	Specific IgE (IU/mL)						Glycinin- derived fragment	WPPE-specific IgE/total IgE (%)
					WPPE	Ara h 1	Ara h 2	Ara h 3	Ara h 6			
51	MM	5	m	2450	1450	790	790	870	730	810	59	
470	MM	4	m	10 800	2190	1370	1170	1840	1160	1490	20	
488	MM	12	f	470	320	160	47	180	75	81	68	
658	MM	11	m	710	23	16	11	25	14	15	3	
92	S	2.5	m	1170	790	380	340	550	260	200	68	
200	S	9	f	3160	350	180	125	250	145	200	11	
314	S	8	m	1540	110	51	44	72	40	70	7	
437	S	16	m	380	180	94	52	150	70	91	47	
445	S	12.5	m	490	160	100	64	160	90	130	33	
577	S	16	f	560	250	120	51	200	80	110	45	
609	S	6.5	f	730	390	250	120	280	190	220	53	
631	S	16.5	f	630	160	90	41	130	53	77	25	
Pool of peanut-allergic patient sera				960	160	88	53	130	72	120	17	
Pool of milk-allergic patient sera				1320	0	0	0	0	0	0	0	

Total and specific serum IgE concentrations are expressed in IU/mL. Symptoms: MM, mild to moderate; S, severe, as defined in 'materials and methods'. f, female; m, male.

were 62.7, 16.67 and 18.05, 14.84 and 11.9 kDa for Ara h 1, the two naturally occurring isoforms of Ara h 2, Ara h 6 and the glycinin-derived fragment, respectively, according to published values [11, 18]. The MW of Ara h 3 was estimated at 130 kDa using gel filtration chromatography. For the whole protein extract, a mean MW of 100 kDa was calculated taking into account the estimated proportion and the measured MW of the different peanut proteins present in the WPPE [19]. The purity of purified allergens was confirmed by silver stain electrophoresis (data not shown).

Determination of total and specific IgE concentrations

The concentrations of total IgE and of IgE specific to WPPE and to purified peanut proteins were determined using the enzyme allergosorbent tests described previously [5, 17]. Briefly, microtitre plates were coated by passive adsorption of WPPE or purified peanut proteins (5 µg/mL in 50 mM phosphate buffer, pH 7.4, for 24 h at 4 °C). After saturation for at least 4 h at room temperature with EIA buffer (0.1 M phosphate buffer, 0.1% bovine serum albumin (BSA), 0.15 M NaCl, 0.01% sodium azide, pH 7.4), 50 µL/well of serial dilutions of individual serum (1/4–1/2500, in EIA buffer) were dispensed. The total IgE concentration was determined using plates coated with an anti-human IgE (clone LE27, kind gift from J. Grassi [20]). A standard curve was determined using the same plate and standard human IgE samples (World Health Organization) at concentrations ranging from 10 to 0.08 IU/mL. After a 24 h incubation at 4 °C and a wash step, a second anti-human IgE antibody (clone BS17, kind gift from J. Grassi [20]) labelled with acetylcholinesterase (AChE) was used as a tracer. After extensive washings, Ellman's reagent was used as an enzyme substrate and the absorbance was measured at 414 nm.

Culture of RBL SX-38 and RBL 2H3 cells and assessment of FcεRI expression

Culture conditions. Experiments were performed with rat mast cells expressing the human FcεRI α, β and γ chains (RBL SX-38) [15], kindly provided by J. P. Kinet and M. H. Jouvin (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA). Geneticin G418 is used to select and maintain human FcεRI-expressing cells. RBL 2H3 cells (non-transfected cell line, kindly provided by U. Blank, Inserm, Unité E-0225, faculté de médecine Xavier-Bichat, Paris, France) were used as negative control. RBL SX-38 cells were grown as described by Wantke et al. [21] in Eagle's minimum essential medium with Earle's salts containing 10% de complemented fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM

L-glutamine and 2.4 mg/mL geneticin G418 (all from Gibco, Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO₂ incubator. Cells were harvested by exposure to 0.25% trypsin, 1 mM EDTA (Gibco) for 2 min at 37 °C, followed by centrifugation (400 g for 5 min) and resuspension in fresh medium. Pre-confluent cells were split twice a week at a 1 : 10 to 1 : 20 ratio. RBL 2H3 cells were maintained like RBL SX-38 cells, except that G418 was removed from the culture medium.

Assessment of human FcεRIα chain expression by flow cytometry. Cellular suspensions of 3.5 × 10⁵ RBL SX-38 or RBL 2H3 (negative control) were incubated for 1 h at 4 °C with 0.1 µg of a mouse anti-human FcεRIα monoclonal antibody (clone AER37 from eBioscience, San Diego, CA, USA) or control isotype, in phosphate-buffered saline (PBS) (Gibco) plus 1% BSA. After three washes in PBS plus 1% BSA, cells were incubated with 0.5 µg of F(ab')₂ fragments of goat anti-mouse IgG (H+L) antibody conjugated to R-phycoerythrin (Jackson ImmunoResearch, Suffolk, UK). After three washes, cells were finally suspended in 500 µL of FACSFlow (BD Biosciences, Franklin Lakes, NJ, USA) and analysed using a Becton Dickinson FACSCalibur flow cytometer (Erembodegem, Belgium) interfaced with CellQuest Software.

Assessment of human FcεRIβ chain expression by Western blotting. RBL SX-38 or RBL 2H3 (negative control) cells were lysed in electrophoretic buffer according to Laemmli [22] (62.5 mM Tris pH 6.8, 1% SDS, 10% glycerol). The protein content of cell lysates was measured using the BCA kit (Pierce, Rockford, IL, USA) and 5 µg of proteins were reduced by heating with 2.5% of β-mercaptoethanol for 10 min at 95 °C. Proteins were then separated by SDS-PAGE (Mini Protean III system, Bio-Rad, Hercules, CA, USA) and either stained (Gelcode[®] Blue Stain Reagent, Pierce) following the manufacturer's instructions or transferred to PVDF membranes. See-Blue[®] Plus 2 Pre-Stained Standard (Invitrogen, Carlsbad, CA, USA) was used as a MW standard. Membranes were saturated in TBST (20 mM Tris, 250 mM NaCl, 0.5% Tween 20) with 5% non-fat dry milk and then incubated overnight at 4 °C with rabbit polyclonal anti-human FcεRIβ antibody (Upstate Biotechnology, Millipore, Bedford, MA, USA) diluted to 1 : 10 000. After washing with TBST, membranes were incubated for 2 h with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) antibody (Jackson ImmunoResearch) diluted to 1 : 25 000. After several washes, membranes were incubated using the ECL West Dura Trial Kit (Pierce) for 1 min and then revealed on X-OMAT films (Kodak, VWR, Fontenay sous bois, France).

Human FcεRIγ chain expression could not be assessed as no specific antibody was commercially available.

Humanized rat basophilic leukaemia cell-degranulation test

IgE immunopurification. Preliminary experiments with pooled and some individual sera demonstrated a low sensitivity and reproducibility of the cell-degranulation test when using untreated sera. Although pre-incubation with RBL-2H3 cells, IgG depletion using protein G or heating with or without glucose (5 min at 56 °C, or 45 min at 56 °C and in the latter case with a final glucose concentration of 2 M to protect IgE from degradation [23]) yielded intermediate results, IgE immunopurification of sera gave the best results, both in terms of reproducibility and sensitivity. IgE antibodies were then immunopurified from all allergic patient sera as follows: anti-human IgE monoclonal antibodies (clones BS17 and LE27) were equimolarly coupled to CNBr-activated Sepharose™ 4B (Amersham Biosciences, GE healthcare, Uppsala, Sweden) at 1 mg/mL according to the manufacturer's instructions. Sera diluted 1:4 (v/v) in coupling buffer (20 mM phosphate buffer, 0.4 M NaCl, 0.1% BSA, pH 8) were then incubated for 4 h at room temperature with the gel. After centrifugation, the supernatant was removed and extensive washes were performed by suspension of the gel in coupling buffer, followed by centrifugation and removal of the supernatants. IgE were then eluted with elution buffer (0.1 M glycine, 0.4 M NaCl, 0.1% BSA, pH 3). The pH was rapidly neutralized after elution by addition of NaOH 2 M. Total and WPPE-specific IgE were quantified in eluted fractions as described above.

Release assay

The release assay was adapted from Dibbern *et al.* [16] and Hoffmann *et al.* [24]. G418-selected RBL SX-38 cells were seeded at 1.2×10^5 cells/mL (100 µL/well) on a 96-well tissue culture plate (Corning, New York, NY, USA) in culture medium without G418 and cultured for 24 h. Cells were then washed with PBS and sensitized by addition of 100 µL/well of immunopurified IgE from individual human sera from peanut-allergic patients, diluted in culture medium without FBS. After a 24-h incubation, cells were washed three times with NaPIPES buffer (25 mM disodium PIPES, 100 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂, 5.6 mM glucose and 0.1% BSA, pH 7). Degranulation was induced by incubating the sensitized cells with serial dilutions of WPPE or purified allergens in NaPIPES buffer (50 µL/well, 37 °C, 45 min). Release was measured by determining the enzymatic activity of β-hexosaminidase in the supernatant: the supernatant was transferred to a 96-well plate (30 µL/well) and 50 µL of 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (1.3 mg/mL in 0.1 M citric acid buffer pH 4.5) was added. After a 1-h incubation at 37 °C, 100 µL of 0.2 M glycine solution pH 10.7 was added and absorbance was measured at 405 nm.

The specificity of the release was assessed by sensitizing the cells with immunopurified IgE from the pool of milk-allergic patient sera, and inducing degranulation with peanut allergens. Sensitization of cells with immunopurified IgE from the pool of peanut-allergic patient sera was used as a positive control and was performed on each experimental plate to ensure the repeatability of the assay.

The total content of β-hexosaminidase was quantified by lysing the cells with 1% Triton X-100. A reference release was measured after activating the sensitized cells with a monoclonal anti-human IgE antibody (clone LE27; 100 ng/mL).

After subtraction of the background release, measured using sensitized but non-activated cells, results were expressed as a percentage of the total content or as a percentage of the reference release. The results of specific degranulations obtained with WPPE and purified allergen were analysed by determining the highest mediator release value (MaxR) and the EC₅₀ (the dose giving 50% of this highest release) for each serum and each allergen by fitting a one-site competition curve using GraphPad Prism software.

Results

Assessment of human FcεRI expression

Expression of the human FcεRIα and β chains was confirmed by flow cytometry and Western blotting, respectively. As shown in Fig. 1a, the human FcεRIα chain was detected on RBL SX-38 cells whereas no expression of human FcεRIα chain was observed on RBL 2H3 cells. Repetitive analysis of RBL SX-38 cells demonstrated that this expression is stable over time. The mean fluorescence measured was 817 ± 49 vs. 5.5 ± 0.4 (mean ± SEM, $n = 5$) for the RBL SX-38 and the RBL 2H3 cells, respectively.

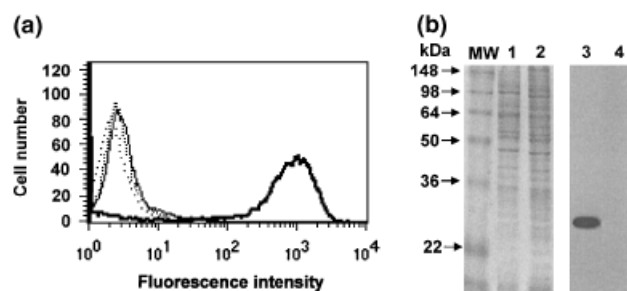


Fig. 1. Expression of human FcεRIα and β chains on RBL 2H3 and RBL SX-38 cells. (a) Expression of human FcεRIα chain measured by flow cytometry: RBL SX-38 cells were incubated with mouse anti-human FcεRIα chain antibody (—) or with isotype control antibody (---), as the same as RBL 2H3 [mouse anti-human FcεRIα chain antibody (—) or isotype control antibody (---)]. (b) Expression of human FcεRIβ chain measured by Western blot: SDS-PAGE of RBL SX-38 (1) and RBL 2H3 (2) cell lysates and corresponding Western blots using anti-human FcεRIβ chain antibody (RBL SX-38 lane 3 and RBL 2H3 lane 4). MW, molecular weight.

The immunoblot of a whole-cell lysate of RBL SX-38 cells revealed a band corresponding to the 28 kDa human Fc ϵ RI β chain, whereas this band was not present in the whole cell lysate of RBL 2H3 cells (Fig. 1b). These results confirmed that under our experimental culture conditions, both α and β chains of the human Fc ϵ RI are stably expressed by RBL SX-38 cells.

Total and specific IgE

No peanut-specific IgE was detected in pooled sera from milk-allergic patients (control sera). Conversely, all of the 12 peanut-allergic patients had IgE against all the tested peanut allergens. The concentrations of total IgE and IgE specific to WPPE, Ara h 1, Ara h 2, Ara h 3, Ara h 6 and glycinin-derived fragment are reported in Table 1. A high variability is observed in the concentrations of specific anti-peanut IgE, which ranged from 23 to 2200 IU/mL, representing 3% to 68% of the total IgE. For all sera, anti-Ara h 3- and Ara h 1-specific IgE had a higher concentration than anti-Ara h 2- and Ara h 6-specific IgE. Interestingly, the anti-Ara h 2- and Ara h 6-specific IgE concentrations were almost similar.

Release assay

Determination of optimal IgE quantity for sensitization of cells. We compared the degranulations obtained when RBL SX-38 cells were sensitized with various dilutions of IgE immunopurified from sera and then activated by the anti-human IgE antibody. As shown in Fig. 2 with four representative sera (i.e. # 51, 92, 470 and 631), the reference release was maximum when using IgE concentrations at 10 IU/mL. The same results were observed with

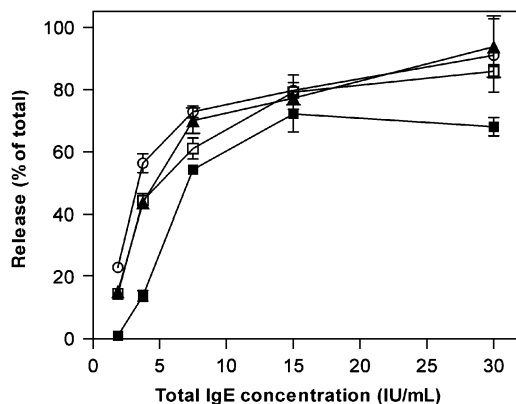


Fig. 2. β -Hexosaminidase release of RBL SX-38 cells sensitized with serial dilutions of sera and activated with anti-IgE monoclonal antibodies. Cells were sensitized with various dilutions of serum 51 ($\square$), 92 ($\circ$), 470 ($\blacktriangle$) or 631 ($\blacksquare$) corresponding to total IgE ranging from 30 to 2 IU/mL, and the release was induced by addition of anti-human IgE monoclonal antibodies. Release is expressed as a percentage of the total cell content of β -hexosaminidase.

all other individual sera (not shown). Sera were then diluted to 10 IU/mL of total IgE for all further studies.

Repeatability and reproducibility. Sensitization of cells with IgE immunopurified from a pool of sera from peanut-allergic patients and degranulation induced by anti-human IgE antibody were performed on several plates either on the same day or on different days. Analysis of 28 tests performed on four different days demonstrated a background release of $4.4\% \pm 0.5$ of total release (mean SEM). The mean reference release obtained was 66% of the total release, with intra- and inter-assay coefficient of variation $< 10\%$, ensuring the reproducibility of the test.

Whole peanut protein extract and purified peanut allergen-specific degranulations

Controls, e.g. RBL SX-38 cells sensitized with the pool of milk-allergic patient sera or RBL 2H3 cells sensitized with sera from peanut-allergic patients, did not result in any significant β -hexosaminidase release after activation with WPPE or purified peanut allergens (data not shown). With all sera from peanut-allergic patients and with all peanut allergens, we observed dose-response curves of degranulation, as shown in Fig. 3 for serum # 445. β -Hexosaminidase release increased in parallel with the allergen concentrations, reaching a peak and then decreasing for a higher allergen concentration. For each serum and each allergen, the highest observed release (MaxR) and the EC50 determined are presented in Table 2. Interestingly, with six individual sera (i.e. # 92, 200, 437, 488, 609 and 631) and with the pool of peanut-allergic patient sera, the degranulation curves obtained after activation by purified Ara h 6 reached a first step and then increased again on increasing the concentrations of allergens. To a lesser extent, similar curves were observed with the same sera when the degranulation was induced by Ara h 2 (not shown).

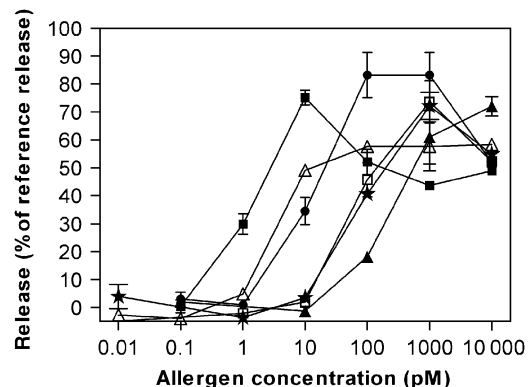


Fig. 3. β -Hexosaminidase release of RBL SX-38 cells sensitized with serum 445 and activated with ranging concentrations of whole peanut protein extract ($\bullet$), Ara h 1 ($\star$), Ara h 2 ($\blacksquare$), Ara h 3 ($\square$), Ara h 6 ($\triangle$) or glycinin-derived fragment ($\blacktriangle$). Release is expressed as a percentage of the reference release.

Table 2. β -Hexosaminidase reference release and maximum release (MaxR) and EC50 values obtained with RBL SX-38 cells sensitized with human sera, and activated with WPPE, Ara h 1, Ara h 2, Ara h 3, Ara h 6 and glycinin-derived fragment

Sera	Symptoms	Reference release	WPPE		Ara h 1		Ara h 2		Ara h 3		Ara h 6		Glycinin-derived fragment	
			MaxR (%)	EC50 (pM)	MaxR (%)	EC50 (pM)	MaxR (%)	EC50 (pM)	MaxR (%)	EC50 (pM)	MaxR (%)	EC50 (pM)	MaxR (%)	EC50 (pM)
51	MM	57	82	2.2	75	190	56	2.3	72	43	66	2.7	68	150
470	MM	63	66	17	85	200	67	2.2	66	100	49	2.9	58	270
488	MM	81	95	9.4	78	8	58	1.5	72	18	56	1.2	71	130
658	MM	85	24	40	18	220	32	3.8	23	170	24	1220	23	3090
92	S	63	98	7	91	74	97	1.3	93	50	47	2.8	95	460
200	S	84	59	18	41	160	35	1.9	44	130	10	2.6	43	950
314	S	69	43	25	43	200	37	2.4	42	180	31	7.9	34	440
437	S	80	79	21	58	230	54	3.2	62	90	30	2.4	56	720
445	S	62	89	13	75	85	80	1.4	76	64	60	3.2	74	260
577	S	69	86	1.2	72	22	65	1.9	68	22	47	3.8	74	200
609	S	55	89	1.3	79	18	48	2.2	82	55	24	0.7	70	150
631	S	66	70	1.5	64	140	71	2	69	65	51	1.8	60	310
Median		67	81	11	74	150	57	2.1	69	65	47	2.8	64	290
Pool of peanut-allergic patient sera		66	69	6.4	60	130	44	2.1	59	140	29	2.7	52	440

MaxR is expressed as a percentage of the reference release obtained with the anti-IgE antibody. The dose giving 50% of this highest release (EC50) is expressed as picomolar. Symptoms: MM, mild to moderate; S, severe.

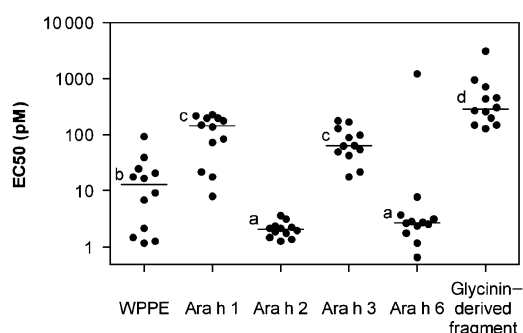


Fig. 4. Comparison of EC50 values obtained with whole peanut protein extract (WPPE), Ara h 1, Ara h 2, Ara h 3, Ara h 6 and glycinin-derived fragment with the 12 individual sera tested. a, b, c and d indicate a significant difference vs. other groups using ANOVA and Tukey's post-test ($P < 0.05$).

The EC50 values obtained using WPPE, Ara h 1, Ara h 3 and glycinin-derived fragment showed a considerable variability within the population studied, whereas EC50 obtained with Ara h 2 and Ara h 6 were more uniform, except for one serum (# 658) (Table 2 and Fig. 4). EC50 triggered by Ara h 2 and Ara h 6 were significantly lower than those obtained after activation by WPPE, Ara h 1, Ara h 3 and glycinin-derived fragment. The medians were 2.1 and 2.8 pM (corresponding to 37 and 41 pg/mL, respectively) for Ara h 2 and Ara h 6, respectively, while for the WPPE, Ara h 3, Ara h 1 and glycinin-derived fragment, the medians were 11, 65, 150 and 290 pM, respectively (corresponding to 1.1, 8.4, 9.4 and 3.5 ng/mL, respec-

tively). This implies that, depending on the serum, 5–100-fold more molecules of Ara h 1 and Ara h 3 are needed to obtain the same release as that obtained with Ara h 2 or Ara h 6.

It is worth noting that the lowest EC50 values were observed with sera characterized by the highest percentage of specific vs. total IgE (i.e. sera # 51, 92, 488, 577 and 609). Conversely, sera that needed higher concentrations of allergens had a low percentage of specific vs. total IgE (i.e. sera # 314 and 658).

For a given serum, we did not observe a considerable variation in the MaxR values obtained after activation by the different allergens. Conversely, for a given allergen, we observed a considerable variability in the MaxR depending on the sera used to sensitize the cells. As an example, MaxR values ranged from 24% to 98% for the WPPE. The MaxR values were found to be positively correlated with the percentage of allergen-specific vs. total IgE in each serum. A significant positive correlation was observed for the specific releases induced by WPPE, Ara h 3, Ara h 1 (Fig. 5) and glycinin-derived fragment ($r^2 = 0.94, 0.84, 0.78$ and 0.69 , respectively). Conversely, this correlation was lower in the case of releases induced by the 2S albumins Ara h 2 and Ara h 6 ($r^2 = 0.49$ and 0.33 , respectively) (data not shown).

Discussion

Humanized RBL cells expressing the whole human Fc ϵ RI receptor provide an appropriate cell-based test to analyse

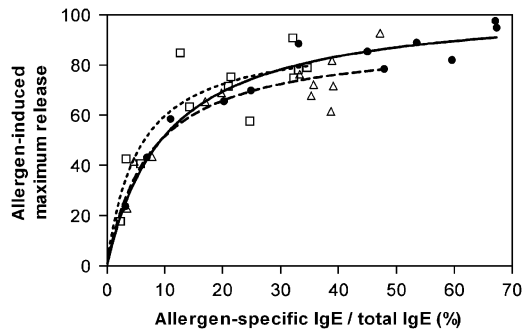


Fig. 5. Evaluation of the correlation between specific vs. total IgE ratio (%) and the maximum release, as observed for whole peanut protein extract (WPPE) (●, —, $r^2 = 0.94$), Ara h 3 (△, ---, $r^2 = 0.84$) and Ara h 1 (□, ..., $r^2 = 0.78$), using 12 sera. Specific vs. total IgE ratio was estimated for each of the different allergen i.e. WPPE, Ara h 3 and Ara h 1. Release is expressed as a percentage of the reference release obtained using anti-IgE monoclonal antibody, as defined in 'materials and methods'.

the functionality of IgE–allergen interactions. It is adapted to the 96-well plate format, reproducible and allows wide and rapid screening of human sera. In contrast to the classic human basophil-degranulation test [25], it does not require fresh blood cells from a patient for each experiment. In the present study, RBL SX-38 cells were sensitized using sera from peanut-allergic patients. After developments to optimize the assay parameters, it was used to compare the capacity of WPPE and major purified peanut allergens, i.e. Ara h 1, Ara h 2, Ara h 3, Ara h 6 and glycinin-derived fragment, to cross-link IgE and trigger the degranulation of the sensitized cells.

We first observed that untreated sera could not be directly incubated with RBL SX-38 cells, confirming that some factors may hamper the binding of IgE to the FcεRI expressed at the membrane of the cells, and adverse effects for the cells were observed [16, 26, 27]. Those factors and how they may act on the cells and interfere with the assay have not yet been identified. However, in the present study, we demonstrated that the IgE-immunopurification of the sera was a simple, rapid and efficient treatment to remove the interfering factors with regard to the background release, the sensitivity and reproducibility of the assay.

Because RBL SX-38 cells stably express the α, β and γ chains of the human FcεRI receptor at relatively high amounts, e.g. up to 130 000 receptors/cell, the assay is very sensitive as compared with assays using other humanized RBL cells [21]. We demonstrated that, whatever the serum, the optimal sensitization of cells was obtained with 1 IU of IgE per well. This corresponds to 1.5–2 IgE molecules per receptor, suggesting that under these experimental conditions, all FcεRI expressed on the surface of the cells may bind an IgE molecule. Under these conditions, using 12 individual sera from peanut-allergic patients to sensitize the cells, we observed reference releases ranging from 55% to 85% of the total cellular

content of β-hexosaminidase after activation with anti-IgE monoclonal antibodies. This reference release was significantly higher than that observed with the same cell line by Dibbern et al. [16], or using classic human basophil-degranulation tests [10]. The results were also better than those obtained using other humanized RBL assays, in particular, those using cells only transfected with the α chain of the human FcεRI [26, 28–30]. It is worth noting that for some sera, we obtained nearly 100% of the reference release after activation with WPPE or purified allergens.

However, a limitation of the cell-based test presented in our study is that only sera with a percentage of allergen-specific vs. total IgE > 2% can be used (not shown).

Using the test under the optimized experimental conditions and characterized sera from peanut-allergic patients (i.e. with known total, WPPE- and allergen-specific IgE concentrations), we compared the degranulation capacity of Ara h 1, Ara h 2, Ara h 3, Ara h 6 and glycinin-derived fragment.

For each serum tested, we observed significant degranulation with all the purified allergens. Although not statistically significant, the medians of the MaxR values were the highest with Ara h 1 and Ara h 3 and correlated with the highest concentrations of specific IgE (Tables 1 and 2). Conversely, the medians of MaxR values obtained with Ara h 6 were the lowest (47% of the reference release). However, the particular degranulation curves obtained with this allergen render the evaluation and interpretation of the MaxR values difficult as release stabilized in the first instance and then increased again for high concentrations of Ara h 6 in a second phase. Such patterns may be explained by the presence and successive mobilization of anti-Ara h 6-specific IgE of decreasing affinity. Ara h 2 and Ara h 6 belong to the same protein family, i.e. 2S albumins, and have conserved structures, which can result in IgE cross-reactivity [12]. Thus, within the human polyclonal specific IgE response, the IgE of the lowest affinity that were mobilized during the second phase of the degranulation could correspond to Ara h 2-specific IgE also recognizing Ara h 6 but with the lowest affinity than Ara h 6-specific IgE.

Dibbern et al. [16] showed that the basophil-degranulation activity of sera from peanut-sensitive patients correlated with the percentage of allergen-specific IgE. In contrast, Marchand et al. [26] found no correlation between the allergen-specific IgE contents and the elicitation potential of serum. Although we observed various release intensities in the studied population, the percentage of specific IgE was found to be a good predictor of the ability of a serum to sensitize the RBL SX-38 cells and to induce an allergen-specific release.

With regard to allergens, a good indicator of their allergenic potency is the dose needed to efficiently induce cell degranulation. In the present study, we calculated

EC50 values for each allergen and each serum. We showed that Ara h 2 and Ara h 6 were the most active peanut allergens in terms of the capacity to cross-link IgE and induce degranulation. Conversely, Ara h 1, Ara h 3 and glycinin-derived fragment were less active because higher doses were required to induce a similar release. Indeed, the median EC50 of Ara h 1 was 70 times that of Ara h 2. This is in line with the results of Palmer *et al.* [31], demonstrating that a more than 33-fold higher dose of Ara h 1 than of Ara h 2 was necessary to obtain the same release. This is also in line with Koppelman *et al.* [10], who found that Ara h 2 was the most potent peanut allergen as compared with Ara h 1 and Ara h 3 using a human basophil-degranulation test. Studies comparing the biological activity of peanut allergens by skin prick tests and IgE reactivity showed that Ara h 2 and Ara h 6 were the most frequently and the most strongly recognized peanut allergens [9–11]. Our results with the RBL SX-38 release assay are in agreement with those clinical observations and, altogether, these studies demonstrate the high allergenic potential of 2S albumins compared with other peanut allergens. This is surprising when considering the proportion of the different allergens in whole peanut, i.e. 14%, 6.5%, 50% and 4.5% of the total protein for Ara h 1, Ara h 2, Ara h 3 and Ara h 6, respectively [19]. Despite the small proportion of 2S albumins in the seeds, these proteins may play a major role in the allergic reaction to peanut.

It could be hypothesized that steric hindrance decreases the accessibility of IgE epitopes in large molecules such as Ara h 1 or Ara h 3 when compared with Ara h 2 and Ara h 6. Conversely, the glycinin-derived fragment is a molecule of the same size range as Ara h 2 and Ara h 6, and it is very immunoreactive in view of its IgE-binding capacity (Table 1). However, the glycinin-derived fragment showed very weak degranulation potential in our assay, suggesting that steric hindrance on its own is not the only reason for the lower biological activity.

Neither MaxR nor EC50 correlated well with the severity of the clinical manifestations of peanut allergy in the patients in our study. The intensity of the release was instead correlated with the amount of allergen-specific IgE vs. total IgE in each patient. This confirms that the capacity to induce mast-cell degranulation cannot completely account for the clinical reactivity, i.e. the capacity of an allergen to elicit an allergic reaction *in vivo*. Furthermore, in this assay, we used IgE immunopurified from sera. Thus, it does not take into account the complexity that occurs *in vivo*, for example the role of some factors like specific IgG that can amplify or dampen IgE-induced mast-cell activation via Fc receptors [32].

In conclusion, the optimized RBL SX-38 release assay is a sensitive and reproducible test for comparing the biological activity of different peanut allergens. The comparison of EC50 values demonstrated that Ara h 2 and Ara h 6 were the most potent peanut allergens compared with Ara

h 1 and Ara h 3. In most cases, the intensity of the release was positively correlated with the relative concentration of allergen-specific IgE in the sera. This assay is also very specific and sensitive enough to evaluate the allergenicity of complex food. It can thus be used to evaluate the effect of thermal or chemical treatments, processing and/or digestion on the capacity of food and food allergens to elicit an allergic reaction because those treatments have been shown to have an impact on the IgE-binding capacity [33, 34].

Acknowledgements

We thank J. P. Kinet and M. H. Jouvin for providing the RBL SX-38 cells and U. Blank for providing the RBL 2H3 cells.

References

- Osterballe M, Hansen TK, Mortz CG, Host A, Bindslev-Jensen C. The prevalence of food hypersensitivity in an unselected population of children and adults. *Pediatr Allergy Immunol* 2005; 16:567–73.
- Moneret-Vautrin DA, Rance F, Kanny G *et al.* Food allergy to peanuts in France – evaluation of 142 observations. *Clin Exp Allergy* 1998; 28:1113–9.
- Bock SA, Munoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 2001; 107:191–3.
- Bock SA, Munoz-Furlong A, Sampson HA. Further fatalities caused by anaphylactic reactions to food, 2001–2006. *J Allergy Clin Immunol* 2007; 119:1016–8.
- Bernard H, Paty E, Mondoulet L *et al.* Serological characteristics of peanut allergy in children. *Allergy* 2003; 58:1285–92.
- Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G, O'Brien T. Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *J Allergy Clin Immunol* 1991; 88:172–9.
- Kleber-Janke T, Cramer R, Appenzeller U, Schlaak M, Becker WM. Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int Arch Allergy Immunol* 1999; 119:265–74.
- Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien TJ, Helm RM. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* 1992; 90:962–9.
- Flinterman AE, van Hoffen E, den Hartog Jager CF *et al.* Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clin Exp Allergy* 2007; 37:1221–8.
- Koppelman SJ, Wensing M, Ertmann M, Knulst AC, Knol EF. 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. *Clin Exp Allergy* 2004; 34:583–90.

- 11 Bernard H, Mondoulet L, Drumare MF *et al.* Identification of a new natural Ara h 6 isoform and of its proteolytic product as major allergens in peanut. *J Agric Food Chem* 2007; 55:9663–9.
- 12 Koppelman SJ, de Jong GA, Laaper-Ertmann M *et al.* Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2. *Clin Exp Allergy* 2005; 35:490–7.
- 13 Koppelman SJ, Knol EF, Vlooswijk RA *et al.* Peanut allergen Ara h 3: isolation from peanuts and biochemical characterization. *Allergy* 2003; 58:1144–51.
- 14 Rabjohn P, Helm EM, Stanley JS *et al.* Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* 1999; 103:535–42.
- 15 Wiegand TW, Williams PB, Dreskin SC, Jouvin MH, Kinet JP, Tasset D. High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I. *J Immunol* 1996; 157:221–30.
- 16 Dibbern DA Jr, Palmer GW, Williams PB, Bock SA, Dreskin SC. RBL cells expressing human Fc epsilon RI are a sensitive tool for exploring functional IgE-allergen interactions: studies with sera from peanut-sensitive patients. *J Immunol Methods* 2003; 274:37–45.
- 17 Mondoulet L, Paty E, Drumare MF *et al.* Influence of thermal processing on the allergenicity of peanut proteins. *J Agric Food Chem* 2005; 53:4547–53.
- 18 Chatel JM, Bernard H, Orson FM. Isolation and characterization of two complete Ara h 2 isoforms cDNA. *Int Arch Allergy Immunol* 2003; 131:14–8.
- 19 van Wijk F, Hartgring S, Koppelman SJ, Pieters R, Knippels LM. Mixed antibody and T cell responses to peanut and the peanut allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 in an oral sensitization model. *Clin Exp Allergy* 2004; 34: 1422–8.
- 20 Grassi J, Didierlaurent A, Stadler BM. Quantitative determination of total and specific human IgE with the use of monoclonal antibodies. *J Allergy Clin Immunol* 1986; 77:808–22.
- 21 Wantke F, MacGlashan DW, Langdon JM, MacDonald SM. The human recombinant histamine releasing factor: functional evidence that it does not bind to the IgE molecule. *J Allergy Clin Immunol* 1999; 103:642–8.
- 22 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- 23 Binaghi RA, Demeulemester C. Influence of the medium on the heat and acid denaturation of IgE. *J Immunol Methods* 1983; 65:225–33.
- 24 Hoffmann A, Jamin A, Foetisch K *et al.* Determination of the allergenic activity of birch pollen and apple prick test solutions by measurement of beta-hexosaminidase release from RBL-2H3 cells. Comparison with classical methods in allergen standardization. *Allergy* 1999; 54:446–54.
- 25 Asero R, Ballmer-Weber BK, Beyer K *et al.* IgE-mediated food allergy diagnosis: current status and new perspectives. *Mol Nutr Food Res* 2007; 51:135–47.
- 26 Marchand F, Mecheri S, Guilloux L, Iannascoli B, Weyer A, Blank U. Human serum IgE-mediated mast cell degranulation shows poor correlation to allergen-specific IgE content. *Allergy* 2003; 58:1037–43.
- 27 Vogel L, Luttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional *in vitro* assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005; 60:1021–8.
- 28 Gilfillan AM, Kado-Fong H, Wiggan GA, Hakimi J, Kent U, Kochan JP. Conservation of signal transduction mechanisms via the human Fc epsilon RI alpha after transfection into a rat mast cell line, RBL 2H3. *J Immunol* 1992; 149:2445–51.
- 29 Lowe J, Jardieu P, VanGorp K, Fei DT. Allergen-induced histamine release in rat mast cells transfected with the alpha subunits of Fc epsilon RI. *J Immunol Methods* 1995; 184:113–22.
- 30 Ladics GS, van Bilsen JHM, Brouwer HMH, Vogel L, Vieths S, Knippels LMJ. Assessment of three human Fc epsilon RI-transfected RBL cell-lines for identifying IgE induced degranulation utilizing peanut-allergic patient sera and peanut protein extract. *Regul Toxicol Pharmacol* 2008; 51:288–94.
- 31 Palmer GW, Dibbern DA Jr, Burks AW *et al.* Comparative potency of Ara h 1 and Ara h 2 in immunochemical and functional assays of allergenicity. *Clin Immunol* 2005; 115:302–12.
- 32 Bruhns P, Frémont S, Daéron M. Regulation of allergy by Fc receptors. *Curr Opin Immunol* 2005; 17:662–9.
- 33 Lucas JS, Cochrane SA, Warner JO, Hourihane JO. The effect of digestion and pH on the allergenicity of kiwifruit proteins. *Pediatr Allergy Immunol* 2007; 19:392–8.
- 34 Scheurer S, Lauer I, Foetisch K *et al.* Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. *J Allergy Clin Immunol* 2004; 114:900–7.