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Gastro-duodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential

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Clinical and Experimental Allergy

Summary

Background The process of gastro-duodenal digestion may play a role in determining the allergenic properties of food proteins. The sensitizing and allergenic potential of digestion products of highly degraded allergens, such as the major peanut allergen Ara h 1, is currently under debate. We evaluated the effect of *in vitro* gastro-duodenal digestion of Ara h 1 on T cell reactivity and basophil histamine release.

Methods An *in vitro* model of gastro-duodenal digestion was used to investigate changes in the allergenic properties of Ara h 1 using *in vitro* assays monitoring T cell reactivity (proliferation, cytokine production) and histamine release of basophils from peanut allergic individuals. The digestion process was monitored using an SDS-PAGE gel.

Results *In vitro* gastric digestion led to rapid degradation of Ara h 1 into small fragments M_r L5600. Gastric digestion did not affect the ability of Ara h 1 to stimulate cellular proliferation. Gastro-duodenal digestion significantly reduced its ability to stimulate clonal expansion ($P < 0.05$; Wilcoxon's signed rank test). The Th-2 type cytokine polarization of T cells from peanut allergic donors (IFN- γ /IL-13 ratio and IFN- γ /IL-4 ratio of CFSE^{low} CD4⁺ T cells) remained unchanged regardless of the level of digestion. Histamine release of basophils from peanut allergic individuals was induced to the same extent by native Ara h 1 and its digestion products.

Conclusion Gastro-duodenal digestion fragments of Ara h 1 retain T cell stimulatory and IgE-binding and cross-linking properties of the intact protein.

Keywords Ara h 1, digestion, food allergy, peanut allergy, T cell reactivity

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Introduction

Peanut allergy is one of the most common and serious immediate type hypersensitivity reactions to foods. Throughout the last decades, the prevalence of peanut allergy has been constantly rising in Europe and United States [1, 2] and has become the major cause of fatal food-induced anaphylaxis [3, 4]. Unlike many other food allergies, it is rarely outgrown [5, 6]. Thus diagnosis means, for most of the patients, lifelong avoidance of peanut allergens and careful attention to hidden peanut containing ingredients [7]. Therefore, an understanding of

the underlying mechanisms in peanut allergy is vital if effective strategies for both prevention and treatment are to be developed in the future.

The routes of sensitization involved in the pathogenesis of food allergy are a controversial point of discussion [8, 9]. Gastro-intestinal sensitization is likely for many food allergens for which resistance to digestion has been considered to be a prerequisite. This has led to resistance to pepsinolysis being used in a predictive mode as part of the allergenic risk assessment process for novel proteins destined for food use [10], although later studies have indicated that the relationship between resistance to digestion and allergenicity may be less clear cut than was originally thought [11]. Recent studies performed on food

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Table 1. Patients' characteristics

ID	Anti-whole peanut IgE (IUA/L)	Anti-Ara h 1 IgE (IUA/L)	Total IgE (kU/L)	Clinical symptoms after peanut consumption	Sensitization to other allergens (RAST Phadia CAP)	Age	Gender	Tests performed
1679	2387	1041	ND	U	C, HDM, S (6), OVA, W (5), F, Al (4)	9	M	IB
1691	106	53	226	A (BO, GI)	GPM (2), HDM (1)	22	M	Prolif; FC
1695	950	622	> 2000	GI	GPM (6), HDM (2)	8	F	IB
2124	340	32	755	A (U, BO)	Al (3), ST (2), M, W, H (1)	6	M	Prolif;
2204	4.5	2.2	> 2000	A (OE, RBP)	C, S, H (3), PN, CN, Al (2)	48	M	Prolif,
2205	45	29	535	A (OE, BO, GI)	H (3), C(2)	25	M	Prolif, FC
2206	106	39	> 2000	A (RBP, OE, GI)	C, W, H, CN (3), E, PN, Al (2)	20	M	Prolif, FC
2207	42	25	ND	A (RPB, GI)	C, W, S, H, PN, CN, Al (2)	28	M	Prolif, FC, HR
2208	18	10	26.9	A (OE, GI)	C (2)	23	M	Prolif, FC
2209	250	91	> 2000	A (RBP, BO)	T, R, B (6), C, D, H, CN (4), PN (3), R, CH, E, W (2)	27	F	Prolif, FC, IB
2210	5	3	87.4	OE	HDM (1)	27	M	Prolif, FC
2211	362	171	1263	A (BO, U)	S, H, Al (4), E (3), W, HDM, PN, CN, ALA (2)	8	F	Prolif
2304	327	262	1013	A (BO, GI)	C, H, PN (3) Al, CN (2)	25	M	Prolif, FC
2305	155	129	> 2000	A (RBP, OE, BO, U) GI	C (4), CN (3), W, PN(2),	18	F	FC, HR
2306	39	34	286	A (RBP, OE, BO, U) GI	B (4)	37	M	FC
2308	17	31	327	OE, GI	S, E (2), H, T (1),	30	F	HR
2551	4.1	ND	298	U, I	BLG, S, CAS (2)	5	M	Prolif

All patients listed displayed a positive SPT to peanut (weal diameter > histamine weal reaction and weal diameter > 3 mm)

A, anaphylaxis (according to criteria listed by Sampson et al. [20]); RBP, reduced blood pressure or associated symptoms of end organ dysfunction; OE, oral oedema; BO, bronchial obstruction; GI, gastro-intestinal symptoms; I, itching of the skin; GPM, grass pollen mix; HDM, house dust mite; ST, strawberry; F, fish; Al, almond; M, milk; BLG, β -lactoglobulin; CAS, casein; W, wheat; C, cat; S, soybean; H, hazelnut; PN, paranut; CN, coconut; E, egg; R, ragweed; T, timothygrass; R, rye; B, birch; D, dog; CH, *Cladosporium herbarum*; ALA, α -lactalbumin; Prolif, proliferation experiments; FC, flow cytometric experiments (CFSE labelling+intracellular cytokine staining), HR, histamine release; U, urticaria; IB, immuno blot.

allergens provided evidence that peptides resulting from extensive digestion still harbour IgE binding and/or T cell activation capacity [12–14]. This might provide an explanation for the high rate of sensitizations to Ara h 1 a major peanut allergen known to undergo rapid enzymatic degradation.

In its native form, one of the major peanut allergens, Ara h 1 is a stable homotrimeric protein comprising several different but closely related subunits, probably held together by inter-twining α -helices at the end of each monomer [15, 16], like other 7S seed storage globulins [17, 18]. These monomer–monomer contact points are the regions where the majority of the 23 IgE-binding epitopes are located [15, 16]. Although intact Ara h 1 is degraded relatively quickly, several lower molecular weight polypeptides persist [15, 19]. While the rapid degradation of the intact monomer and appearance of fragments possessing IgE-binding epitopes during proteolysis upon digestion with various enzymes has been described [15], it has not been performed with enzyme combinations and surfactants found during the gastric and gastro-duodenal digestion *in vivo*. In the case of crude peanut extracts, it has been reported that extensive pepsin digestion leads to loss of IgE binding epitopes [13] whereas another publication using a different experimental setting reported digestion fragments with IgE reactivity [15]. However, the

allergenic properties of Ara h 1 digestion products generated with a system designed to mimic human digestion, have yet to be evaluated.

Therefore, we have used an *in vitro* model designed to mimic closely human gastro-duodenal digestion to monitor changes of Ara h 1 during the course of the digestion process. SDS-PAGE, T cell reactivity (proliferation, cytokine production) and histamine release in basophils from peanut allergic individuals were used to show that gastro-duodenal digestion fragments of Ara h 1 retain T cell stimulatory and allergenic properties.

Methods

Patients' blood samples

Individuals with a doctor diagnosed peanut allergy, a convincing history of peanut anaphylaxis (severe, potentially fatal, systemic allergic reaction that occurred suddenly after contact with peanut [20]) or clear peanut-related symptoms (urticaria, itching of the skin, gastro-intestinal symptoms) within the last 12 months (Table 1), were enrolled after informed consent. Re-evaluation via interview was performed to document the association between exposure to peanut and thereof resulting clinical symptoms. Peanut sensitization was assessed by skin prick

testing (weal diameter > histamine weal reaction and weal diameter > 3 mm) and by measurement of specific IgE-antibodies to peanut extract (CAP/Phadia) and Ara h 1 with an in house ELISA [21] (Table 1). Individuals with no history of peanut allergy and no sensitization (no peanut or Ara h 1 specific IgE, SPT negative) were used as controls. Peripheral blood was processed according to standard procedures. Sera were stored at -20°C and peripheral blood mononuclear cells (PBMCs) used for experiments as described below.

Purification of Ara h 1

Hexane defatted peanut meal [a Virginia red variety (*Arachis Hypogaeae*, L.ssp. *hypogaeae*) peanuts, kindly provided by Dr Shri Sathe, Florida State University, FL, USA] was extracted in water (5 : 1 w : w water to meal, 1 h, 18°C) containing protease inhibitors (Roche complete mini protease inhibitor tablet, Roche, Sussex, UK). After clarification by centrifugation (30 000 g 30 min) ammonium sulphate was added to 70% saturation at 4°C . The soluble fraction was collected by centrifugation (30 000 g 30 min), dialyzed against buffer (Tris, 20 mM, pH 7.5, 150 mM NaCl) and loaded onto a column of Con A Sepharose (Amersham Biosciences, Buckinghamshire, UK). Unbound protein was removed by washing in the same buffer, and the pure Ara h 1 was eluted with the addition of 0.5 M methyl α -D-mannopyranoside. Purity was assessed by reverse phase high-performance liquid chromatography, reflected in a single peak. The protein was dialyzed against 150 mM NaCl and stored frozen in aliquots until required.

In vitro gastro-duodenal digestion

Digestions were performed essentially as described by Moreno et al. [22] and monitored via SDS-PAGE under reducing conditions on a 12% Bis-Tris gel with MES running buffer and stained using a Coomassie-based stain (Invitrogen, Paisley, UK). With the gastric digesta an immunoblot staining using three sera was performed to exclude the possibility that intact protein might be responsible for the measured reactivity as described in detail by Mondoulet et al. [23].

In vitro gastric digestion

Briefly Ara h 1 (2.5 mg/mL in 0.15 M NaCl) was adjusted to a pH 2.5 with 1.0 M HCl and a solution of pepsin (Sigma, Dorset, UK) was added to give approximately 162 U of activity (calculated using the declared activity of the enzyme) per milligram of protein. Samples were placed in a shaking incubator at 37°C , 200 rpm and aliquots of digesta removed before the addition of pepsin at various intervals up to 120 min of digestion. The pH was raised 7.0–7.5 to irreversibly inactivate pepsin by using either

0.5 M ammonium bicarbonate or for large-scale digestions 1.0 M NaOH. Large-scale digestions were used for all subsequent *in vitro* cellular assays. Control samples with no added pepsin were also prepared.

In vitro duodenal digestion

The pH of gastric digesta (120 min) was adjusted to 6.5 with 1.0 M HCl and the following solutions were added: (1) a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate (Sigma) and glyco-deoxycholic acid (Calbiochem, San Diego, CA, USA); (2) 1 M CaCl_2 ; (3) 0.25 M Bis-Tris, pH 6.5 (Sigma); (4) porcine pancreatic lipase (20 μL : 10 mL of total volume) (0.1%, w : v, activity 25 600 U per mg of protein); (5) porcine colipase (40 μL : 10 mL of total volume) (0.055%, w : v). Finally, solutions of trypsin [0.1%, w : v, activity: 13 800 U/mg of protein using α -benzoyl-L-arginine ethyl ester (BAEE) as substrate] and α -chymotrypsin [0.4%, w : v, activity: 44 U/mg of protein using benzoyl-L-tyrosine ethyl ester (BTEE) as substrate] in 0.15 M NaCl at a pH 7.0 were prepared and added at approximately physiological ratios of Ara h 1 : trypsin : chymotrypsin = 1 : 0.0025 : 0.01 (w : w). This gave a final duodenal digestion mix as follows: Ara h 1, 2.3 mg/mL; bile salts, 7.4 mM; CaCl_2 , 9.2 mM; Bis-Tris, 24.7 mM. The digestion was performed at 37°C , and aliquots were taken at various time points (0, 1, 2, 4, 8, and 16 min) for further analysis. The digestion was stopped by adding a solution of Bowman–Birk trypsin–chymotrypsin inhibitor from soybean (Sigma) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix. Ara h1 subjected to 120 min of gastric digestion followed by 16 min of duodenal digestion was used for all *in vitro* assays of allergenic activity as this represents approximately the transit time down the small intestine to the site of the first Peyer's patches.

Proliferation experiments

PBMCs were isolated from venous blood of peanut allergic and non-allergic controls by density gradient centrifugation using Ficoll-PaqueTM Plus (Amersham Biosciences, Uppsala, Sweden) and stored at -190°C . PBMCs (5×10^4 / 200 μL) were cultured in serum-free medium (Ultra culture medium; Bio Whittaker, Walkersville, MD supplemented with 2 mM L-glutamine and 170 mg/L gentamycin sulphate, both from Sigma Science, St Louis, MO, USA). In the presence of different antigen concentrations, cells were incubated in triplicates at a humidified atmosphere with 5% CO_2 for 7 days. IL-2 (25 IU/mL) was used as a positive control, and medium alone served as a negative control. At day 6, tritiated thymidine ($[\text{H}^3]$) (0.5 μCi per well; Amersham Biosciences) was added and, after 16 h of

incubation, incorporated radioactivity (counts per minute) was measured by liquid scintillation.

Peripheral blood mononuclear cells preparation, CFSE labelling, and intracellular cytokine measurement

Thawed cells were resuspended in Hanks balanced salt solution (HBSS) containing 20 mM HEPES and labelled with CFSE (Molecular Probes Inc., Eugene, OR, USA) (5 μ M) for 10 min at 37 °C with gentle shaking. Then HBSS+HEPES containing 4% autologous plasma was added, and excess dye was removed by washing. CFSE-labelled cells were cultured with or without antigens in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 85 mg/L gentamycin-sulphate and 5% autologous plasma in 24-well flat bottom plates at a concentration of 2×10^6 PBMCs/mL medium per well.

On day 7, the percentage of cytokine producing T cells was analysed by intracellular cytokine detection. Expression of the Th1-type-cytokine INF- γ , the Th2-type-cytokines IL-13, IL-4 and the regulatory cytokine IL-10 was assessed with mAb and four-colour flow cytometry (FACSCalibur; Becton Dickinson, San José, CA, USA). Cells were stimulated with 10 ng/mL phorbol myristate-acetate and 1.25 μ M ionomycin, in the presence of 5 μ g/mL Brefeldin A (all from Sigma Chemical Co. Bio Sciences, St Louis, MO, USA) for 4 h. Thereafter, cells were harvested, washed, fixed with 2% formaldehyde and permeabilized (0.2% Saponin). Four-colour fluorescence staining was performed using fluorochrome-labelled rat or mouse anti-human antibodies specific for human surface markers (CD3, CD4) or cytokines (INF- γ , IL-13, IL-10; all from Becton Dickinson) and the respective isotype controls. For all flow cytometric experiments, a total of 10^5 cells was analysed. Data were analysed using Cell Quest Pro Software (Becton Dickinson).

Histamine release assays

A crucial step in immediate type hypersensitivity reactions is cross-linking of membrane bound IgE on basophils and subsequent mediator release. In order to assess the allergenic potential of generated digestion products histamine release assays were performed on Dextran-isolated blood basophils essentially as described [24]. Basophils from two healthy controls and three allergic donors were enriched by Dextran sedimentation, washed and resuspended in histamine release buffer (HRB; Immunotech, Marseille, France). In typical experiments, cells (3×10^6 /mL) were incubated with various concentrations of Ara h 1 or the digestion products (0.1 ng/L–10 μ g/mL) in 96-well microtiter plates (TPP, Trasadingen, Switzerland) for 30 min (37 °C). After incubation, cells were centrifuged at 4 °C for 10 min. Then, cell-free supernatants were recovered, and histamine was measured by a com-

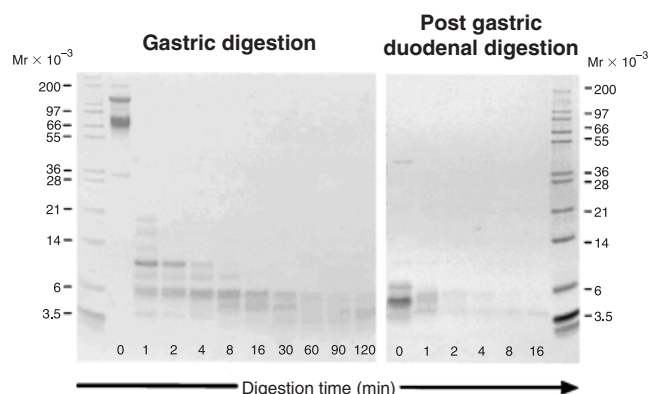


Fig. 1. SDS-PAGE analysis of gastric and post-gastro duodenal digestion time courses of Ara h 1. SDS-PAGE analysis was performed in the presence of reducing agent.

mercial radioimmunoassay (RIA; Immunotech). Histamine release was expressed as percentage of total (extracellular+cellular) histamine measured in lysates of cells that were prepared by freeze thawing.

Results

In vitro gastro-duodenal digestion

During gastric digestion intact Ara h 1 was rapidly degraded with a loss of the M_r 74 500 polypeptide, which was accompanied by the appearance of a series of fragments of M_r 6–19 000 together with the very low molecular weight material running at the gel dye front appearing after 1 min (Fig. 1). The bands were also rapidly digested, with only a broad band around M_r 6000 remaining after 8 min digestion, while at the same time lower molecular weight polypeptides were formed (5.6, 4.5 and 2.8 kDa), which remained stable for 2 h gastric digestion, around the time taken for gastric digestion *in vivo* [25]. The non-occurrence of high molecular peptides with IgE-binding epitopes after the gastric digestion process was further assured via immunoblots using very high loading doses (Fig. 2).

Gastric digesta were then subjected to a stimulated duodenal digestion, which resulted in a very rapid loss (after 1 min) of any polypeptides remaining following gastric digestion. Digestion was continued for 15 min, as this is approximately the transit time down the small intestine to the site of the first Peyer's patch.

Cellular proliferation and cytokine production

To evaluate the potency of the generated digestion products to induce clonal expansion in antigen specific T cells, proliferation experiments were performed with PBMCs from peanut allergic individuals. Proliferation was assessed via measurement of the incorporated

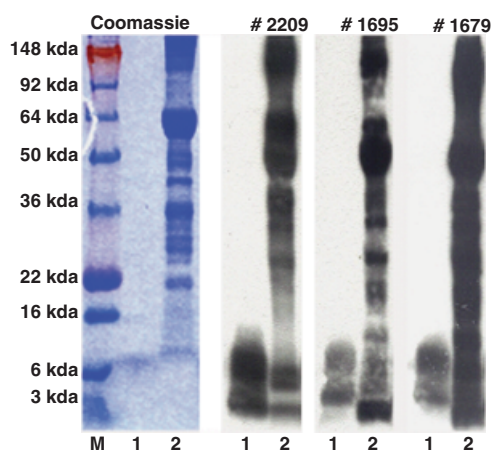


Fig. 2. SDS-PAGE and immunoblot of Ara h 1 using serum from peanut-allergic individuals. Electrophoretic pattern of Ara h 1 gastric digesta (1) and Ara h 1 control (2) (400 µg of the digesta/protein was loaded of each sample) and molecular-weight markers. Immunoblots were performed with three different sera (#2209, #1695, #1679). Sera were diluted to 1/200 in Tris-buffered saline containing Tween 20 supplemented with 5% milk powder.

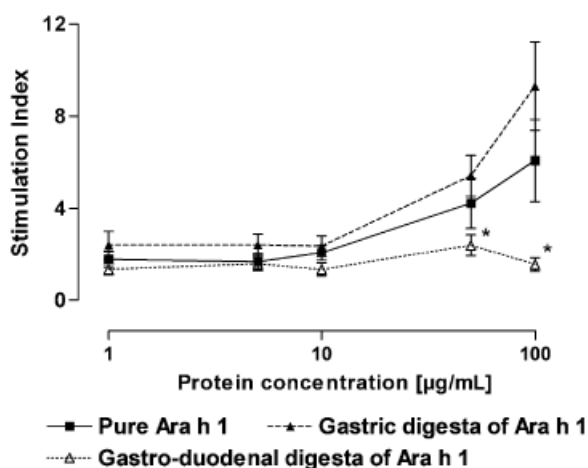


Fig. 3. Peripheral blood mononuclear cells were cultured in the presence of different antigen concentrations for 7 days. Antigen concentration of the digesta (µg/mL) corresponds to the concentration of intact Ara h 1 applied at the beginning of the digestion process. Data represent the mean and SEM of 9 (1–10 µg/mL) or 11 (50 and 100 µg/mL) independent experiments from different peanut allergic individuals (see Table 1). Significance was determined by Wilcoxon's signed rank test (* $P < 0.05$).

radioactive-labelled [H^3] thymidine [26]. As shown in Fig. 3, gastric digestion of Ara h 1 did not reduce the capacity to induce clonal expansion, whereas gastro-duodenal digestion products stimulated significantly less vigorous cellular proliferation. These results were reproducible in all samples reflecting a reduction in T cell stimulating capacity of gastro-duodenal digestion products. However, the proliferative response towards Ara h 1 digestion products was not completely abolished in all samples. Neither gastric nor duodenal fluid alone induced clonal expansion or non-specific inhibition (data not shown).

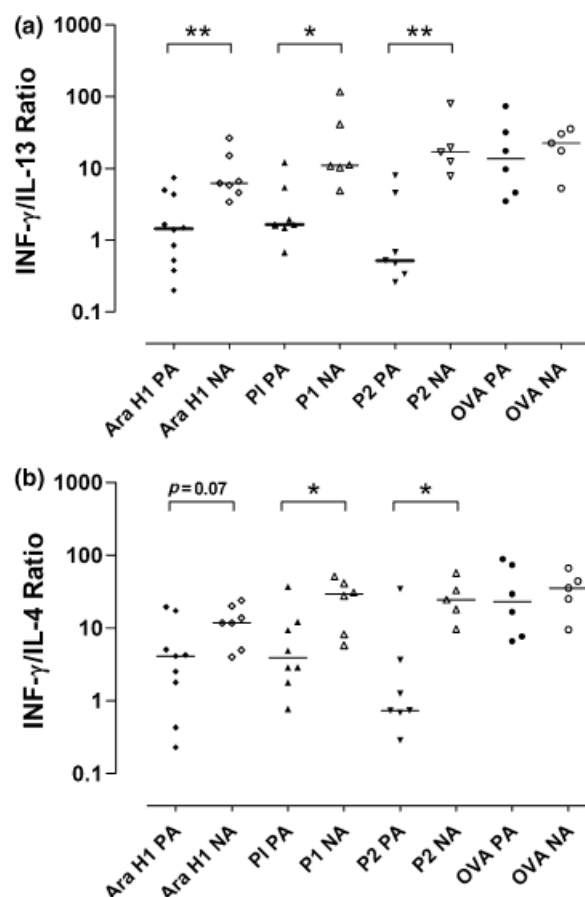


Fig. 4. T helper subset polarization of antigen-specific T lymphocytes from peanut allergic (PA) and non-allergic individuals (NA). Peripheral blood mononuclear cells were CFSE-labelled and co-cultured in the presence of antigens (P1, gastrically digested Ara h 1; P2, gastro-duodenally digested Ara h 1; OVA, ovalbumin) for 7 days. Subset skewing was determined by calculation of Th1/Th2 ratios using percentages of cytokine-producing $CD4^+$ CFSE^{low} cells. Significance was determined by Mann-Whitney U -test (* $P < 0.05$, ** $P < 0.01$).

In recent publications, CFSE staining has been proven to serve as a valuable tool to gate on antigen-specific cells in general [27] and on peanut-specific cells in particular [28]. Therefore, PBMCs from peanut allergic donors were labelled with CFSE and then cultured in the presence of Ara h 1, the digesta therefrom, the respective control antigens and medium alone as described in detail by Turcanu et al. [28]. The results were expressed as percentages of cytokine-positive cells among the antigen-specific T helper cells (CFSE^{low} $CD4^{pos}$). As documented in Fig. 4, the CFSE^{low} $CD4^{pos}$ population of PBMCs from peanut allergic individuals displayed a marked Th-2 polarized cytokine response expressed as the ratio of IFN- γ /IL-13 and IFN- γ /IL-4. Cytokine ratios did significantly differ between peanut allergic and non-allergic individuals. In accordance with published data supporting the specificity of the detected signals, no difference between the different groups (peanut allergic vs. non-

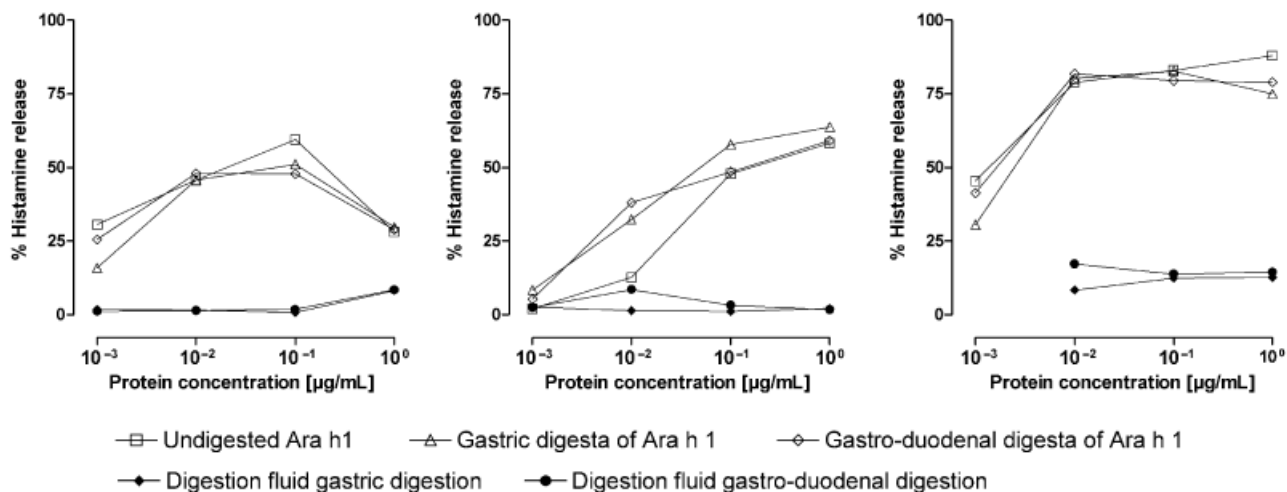


Fig. 5. Histamine release of basophils from three peanut allergic donors stimulated with Ara h 1 and the digestion products derived thereof is expressed as percentage of total (extracellular+cellular) histamine measured in lysates of cells from allergic donors. Antigen concentration of the digesta corresponds to the concentration of intact Ara h 1 applied at the beginning of the digestion process.

allergic) were observed when the clinically irrelevant food allergen ovalbumin was used as a stimulant (Fig. 4). IL- 10^{+} CFSE^{low} CD4⁺ cells were only measurable at very low frequencies, and their relative frequency was neither affected by the type of allergen (Ara h 1 vs. OVA) nor by the degree of digestion of Ara h 1. The Th-2 bias in the proliferating T cell population was even more pronounced (IFN- γ /IL-13-ratio IFN- γ /IL-4) with increasing Ara h 1 digestion (Fig. 4). Based on these results, it seems very likely that the peptides generated by digestion harbour immunoreactive capacities.

Basophil histamine release

To define the biological relevance of IgE binding by the digestion products, histamine release assays were performed. The results obtained demonstrated dose-dependent histamine release curves for both gastro and gastro-duodenal digesta, and the positive controls (intact Ara h 1) as demonstrated in Fig. 5. Negative controls (i.e. digestion media alone) were shown not to induce histamine release. In non-allergic controls, neither the peanut allergen nor the digestion products lead to a significant release of histamine from basophils (< 10% of total histamine). These results demonstrate that Ara h 1 breakdown products are as potent as the intact allergen molecule in stimulating histamine release by basophils. Non-specific effects could clearly be ruled out on the basis of results with control samples.

Discussion

In this study, we describe the persistence of allergenic properties of the major peanut allergen Ara h 1 after simulated gastro-duodenal digestion by means of T cell

activation and IgE-cross-linking experiments. These digested molecules retain an allergenic potential similar to the unaltered protein.

The route of sensitization to food allergens, in general, and to peanut allergens, in particular, is not well defined and several hypotheses have been proposed [8, 9, 29, 30] including the gastro-intestinal route of sensitization. Resistance to digestion, including pepsin, is a property common to many food allergens and is classically considered to be mandatory for this route of sensitization. This has been described for the 2S albumin Ara h 2, as a major peanut allergen belonging to the prolamin superfamily, which has been demonstrated to be remarkably stable in simulated gastric fluid [31]. However, it is apparent there is no absolute correlation between allergenicity and resistance to pepsinolysis [11] and Ara h 1. It is an important peanut allergen and it has been found to be susceptible to proteolysis [13, 19]. We used an *in vitro* model simulating *in vivo* gastro-duodenal digestion more closely than the normally applied pepsin resistance test to monitor changes of Ara h 1 along the enzymatic digestion process in order to define how digestion affected the allergenic properties of Ara h 1.

Gastric digestion lead to a rapid degradation (within minutes; Fig. 1) of the Ara h 1 subunit, which is in accordance with recently published data [13, 19]. By continuing the digestion with duodenal enzymes (simulating the *in vivo* situation), the Ara h 1 peptides remaining after gastric digestion were further broken down (Fig. 1).

To assess the functional properties of the gastro-duodenal digestion products, their potential to elicit T cell responses (cellular proliferation, Th1/Th2 cytokine ratios) was analysed. Gastric digestion of Ara h 1 did not affect cellular proliferation (Fig. 3), again consistent with the

observations of Hong et al. [13], who studied pepsin digestion of whole peanut extract. However, gastric followed by duodenal digestion substantially reduced the amount of clonal expansion, although the cellular response was not completely abolished.

By characterizing the phenotype of the T cells stimulated by either undigested Ara h 1 or its digestion products using intracellular cytokine staining, the persistence of an allergen-specific Th-2 biased cytokine polarization was apparent (IFN- γ /IL-13 ratio and IFN- γ /IL-4 ratio of CFSE-low CD4⁺ T cells). This polarization was consistently present regardless of the extent of digestion (Fig. 3). An allergen-specific Th2-type response has been described previously using peanut-specific T cell clones derived from peanut allergic individuals [32, 33]. A more recent publication described that T cells from peanut allergic individuals display a pronounced Th2-biased allergen-specific T cell response as compared with non-allergic children or peanut-allergic children who became tolerant [28]. The reduced proliferative response argues for the activation of a low number of allergen-specific T cells, although these cells apparently still harbour the allergy driving Th2 phenotype. These observations strengthen the hypothesis that peanut-derived digestion fragments resulting from gastro-duodenal digestion have the potential to maintain and possibly also to induce a sensitization process.

Concomitantly, IgE-mediated mechanisms are considered to be crucial in the initiation of anaphylaxis via cross-linking of membrane bound IgE on mast cells and basophils leading to degranulation of preformed mediators like histamine. Regardless of the degree of digestion (unaltered, gastric, gastro-duodenal), the potential of the digestion products to induce histamine release from basophils of peanut-allergic individuals remained unaffected (Fig. 5). This observation is remarkable as all samples used in these studies were derived from the same initial amount of Ara h 1, rather than from protein concentration *per se*. One explanation for the allergenic properties of these digestion products is that some IgE-binding epitopes of Ara h 1 recognized by patients' sera have to be unmasked by digestion to express their IgE and T cell activating abilities, normally being 'hidden' within the interior of the Ara h 1 monomer. The revealing of these IgE-binding epitopes by the digestion process may compensate for the loss of other IgE-binding epitopes, which may be destroyed by enzymatic cleavage. This observation may provide an additional explanation why peanut proteins are such a potent food allergens irrespective of digestion of the intact protein via pepsin, as it occurs in case of Ara h 1 and is not restricted to the T cell epitope repertoire as described for bet v1-related food allergens [14]. However, the nature of the Ara h 1 degradation fragments remains to be characterized in more detail at a molecular level.

Resistance to digestion and, particularly, to pepsin digestion is still a relevant issue in the weight of evidence approach to assess the allergenicity of a novel protein [34, 35]. Our data support the idea that 'resistance to digestion' is a key feature of allergenic properties. But in addition to this persistence of antigenic properties after enzymatic breakdown should be considered as another key feature of relevant allergens. This strengthens the importance of a broad analytical and immunological approach to cover a more complete picture of what allergenicity is like to meet the demanded standards for allergenicity testing.

In summary, despite the fact that Ara h 1 is broken down by gastro-duodenal digestion into low molecular weight peptides, these molecules retain an allergenic potential in the absence of the intact protein.

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