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An unfolded variant of the major peanut allergen Ara h 2 with decreased anaphylactic potential

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Summary

Background—Peanut allergy causes severe type 1 hypersensitivity reactions and conventional immunotherapy against peanut allergy is associated with a high risk of anaphylaxis.

Objective—Our current study reports proof of concept experiments on the safety of a stably denatured variant of the major peanut allergen Ara h 2 for immunotherapy. We determined the impact of structure loss of Ara h 2 on its IgE binding and basophil degranulation capacity, T cell reactivity as well as anaphylactic potential.

Methods—The secondary structure of untreated and reduced/alkylated Ara h 2 variants was determined by circular dichroism spectroscopy. We addressed human patient IgE binding to Ara h 2 by ELISA and Western blot experiments. RBL-SX38 cells were used to test the degranulation induced by untreated and reduced/alkylated Ara h 2. We assessed the anaphylactic potential of Ara h 2 variants by challenge of sensitized BALB/c mice. T cell reactivity was investigated using human Ara h 2-specific T cell lines and splenocytes isolated from sensitized mice.

Results—Reduction/alkylation of Ara h 2 caused a decrease in IgE binding capacity, basophil degranulation and anaphylactic potential *in vivo*. However, the human T cell response to reduced/alkylated and untreated Ara h 2 was comparable. Mouse splenocytes showed higher metabolic activity upon stimulation with reduced/alkylated Ara h 2 and released similar IL-4, IL-13 and IFN γ levels upon treatment with either Ara h 2 variant.

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Conclusions and Clinical Relevance—Reduced/alkylated Ara h 2 might be a safer alternative than native Ara h 2 for immunotherapeutic treatment of peanut allergic patients.

Keywords

alkylation; anaphylaxis; food allergy; human T cells; hypoallergen; immunotherapy; mouse model; splenocytes; unfolded peanut allergen Ara h 2

Introduction

Food allergy has become a major health problem worldwide. At the moment, allergen specific and nonspecific therapeutic approaches for the treatment of IgE-mediated food allergy are being developed [1]. Allergies can be treated by non-specific approaches like application of anti-IgE-antibodies [2, 3], probiotics [4] or herbal mixtures [5]. The most important therapeutic option is specific immunotherapy (SIT) based on oral, sublingual or subcutaneous immune stimulation with allergens [1]. Successful immunotherapy is associated with the induction of allergen-specific IgG, particularly IgG4 antibodies, which block IgE–allergen binding. Also, SIT-induced IgG antibodies likely directly block effector cell degranulation through interaction with inhibitory Fcγ receptors. However, the results of several independent studies investigating this mechanism are controversial [6–9]. Furthermore, effective treatment leads to increased levels of allergen-specific regulatory T cells (T_{regs}) and T_{helper}1 (T_H1) cells [10, 11].

One major drawback of SIT is that therapeutic allergen application can lead to IgE-mediated allergic reactions in hypersensitive individuals. This adverse side-effect is often related to the tertiary structure of the applied allergen and the presence of conformational IgE epitopes. Immunization strategies using recombinant hypoallergens (for instance defective in IgE binding due to point mutations in IgE epitopes) have been developed to reduce the risk of anaphylactic reactions during SIT (reviewed in [12]). Such engineered allergens often have decreased potential to activate effector cells, but retain the beneficial capacity to stimulate T cells [13, 14]. Allergy against peanut (*Arachis hypogaea*) is one of the most common and severe type I food allergies with more than 1% of children affected in westernized countries including the United States [15], Canada [16], Australia [17] and the United Kingdom [18]. Safe subcutaneous SIT for treatment of peanut allergy or other IgE-mediated food allergies is currently not available due to the high risk of anaphylaxis [12, 19]. Ara h 2 is the most widely recognized and potent allergen for peanut-allergic individuals [20–22]. We and others have recently reported that heating leads to unfolding of the major peanut allergen Ara h 2 *in vitro* [23, 24]. However, this unfolded Ara h 2 variant still contains linear IgE epitopes, and conformational epitopes might form again upon refolding *in vivo*. Alkylation of cysteine sulfhydryl groups is widely used in proteomics for the generation of irreversibly reduced, linearized proteins [25]. Recently, this method has been reported as a useful tool for the generation of a hypoallergenic variant of the major peach allergen Pru p 3 [26]. Another study utilized an alkylated form of Ara h 2 for the identification and investigation of linear IgE epitopes and subepitopes [27]. We apply this concept here to stabilize the unfolded state of Ara h 2 and investigate the allergenic and anaphylactic potential of this variant. Our

results provide evidence that stably unfolded alkylated Ara h 2 is a promising therapeutic alternative to native Ara h 2 for SIT of peanut allergy.

Materials and methods

Allergens

Ara h 2 was purified as previously described with minor modifications [23]. A detailed protocol can be found in the Material and methods section of the online supplement. Ara h 2 peptides (GenScript USA Inc., Piscataway, NJ, USA) were prepared to > 90% purity, reconstituted at 2 mg/ml in PBS and stored in aliquots at -80°C until use. Human serum albumin (HSA) and ovomucoid were obtained from Sigma Aldrich (Vienna, Austria).

Reduction and alkylation

Proteins were reduced and alkylated according to a published protocol [25]. Briefly, 1 mg protein/ml 100 mM $(\text{NH}_4)_2\text{CO}_3$ was mixed 1 : 1 with a 2 x alkylation cocktail (acetonitril containing 0.5% triethylphosphin and 2% iodoethanol, pH 10,0; all reagents obtained from Sigma) yielding a final concentration of 0.5 mg/ml. After 60 min of incubation at 37°C , samples were extensively dialysed against H_2O , snap frozen in liquid N_2 and stored at -80°C until use.

Circular dichroism

Circular dichroism (CD) spectroscopy was performed as previously described [28].

Briefly, measurements were performed with a J-715 spectropolarimeter (Jasco, Gross-Umstadt, Germany) at 0.1 mg/ml H_2O using a 1-mm path length quartz cuvette (Hellma, Müllheim, Germany) equilibrated at 20°C . Spectra were measured at a scan speed of 50 nm/min and 0.2 nm resolution. The average of three scans was taken as result after subtraction of the baseline spectrum. The results are shown as mean residue ellipticity (θ) at the respective wavelengths.

Patient recruitment and ethics

The sera of US peanut-allergic patients were provided by the Food Allergy Resource Initiative with our serum sample request being kindly supported by Professors Hugh Sampson and Scott Sicherer, Mount Sinai School of Medicine, New York, USA. This is a voluntary serum repository of individuals with well-characterized food allergies (funded by the Food Allergy Initiative). The participants (or their parents) provided written informed consent and the programme was approved by the Mount Sinai Program for the Protection of Human Subjects of the Mount Sinai School of Medicine. The use of the serum samples was approved by the Ethics committee of the Medical University of Vienna (approval number 771/2011).

ELISA

Ara h 2-specific IgE (human and mouse serum) and IgG (mouse serum) antibodies were detected by ELISA using a standard protocol (please see the material and methods section of the online supplement for details). Cytokine (mouse IL-5, IL-13 and $\text{IFN}\gamma$) contents in

splenocyte supernatants were analysed using ELISA Ready-SET-Go kits (eBioscience, Vienna, Austria) according to the manufacturer's instructions. Apart from IFN γ determination in supernatants of Concanavalin A-stimulated cells (1 : 5 dilution), supernatants were diluted 1 : 1 in assay diluent for analysis.

Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing conditions. Methodological details of the Western blot protocol can be found in the material and methods section of the online supplement.

Degranulation assay

Details of the basophil degranulation assay can be found in the material and methods section of the online supplement. Briefly, RBL-SX38 cells were sensitized overnight with human serum, 1 : 10 diluted in culture medium. Sensitized cells were stimulated with 1 μ g/ml [this concentration induces highest degranulation as determined by dose-response experiments (data not shown)] untreated or reduced/alkylated Ara h 2 in Tyrode's buffer for 1 h at 37°C. Degranulation was assessed by measurement of released β -hexosaminidase in the supernatant and of unreleased enzyme in the respective cell lysate. The presented results were calculated as percentage release of total β -hexosaminidase content.

Mouse anaphylaxis

Female BALB/c mice, 4–6 weeks of age, were obtained from Charles River Laboratories (Willmington, MA, USA) and treated according to European Union rules of animal care with the permission of the Austrian Ministry of Sciences (BWMF-66.009/01870-II/10b/2009). After induction of Ara h 2-specific IgE, 10 weeks after the initial immunization (the treatment protocol is described in detail in the material and methods section of the online supplement), mice were challenged intravenously with 100 μ g of untreated or reduced/alkylated Ara h 2 or reduced/alkylated ovomucoid (negative control) in saline or with saline alone. The animals were monitored for anaphylactic symptoms during 1 h. Body temperature was measured using a rectal thermometer (Bioseb, Vitrolles, France) directly before the challenge and after the stimulation in indicated intervals for 1 h. Animals were subsequently killed by CO₂ application and blood was collected by cardiac puncture. Serum was stored at –80°C until further processing. Serum (1 : 30 diluted in assay diluent) levels of mouse mast cell protease-1 (mMCP-1) were measured in triplicates using the mMCP-1 ready-SET-go kit (eBioscience, Vienna, Austria).

Splenocyte stimulation

Spleen cells were isolated as described in detail in the material and methods section of the online supplement. Splenocytes were seeded in triplicates (4×10^5 cells/well) in 96-well round-bottom plates (Corning). Next, 100 μ l of stimulant (plain complete growth medium or containing 5 μ g/ml concanavalin A, 50 μ g/ml untreated or reduced/alkylated Ara h 2) were applied. Cell supernatant was harvested after 72 h of incubation at 37°C and stored at –80°C until further investigations.

Splenocyte metabolic activity

After counting, splenocytes were resuspended at 4×10^6 cells/ml complete growth medium and seeded in triplicates of 100 μ l into 96-well flat-bottom plates (Corning). A volume of 100 μ l of plain medium or containing stimulants was added as described above. XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2//tetrazolium hydroxide] (Sigma) was solubilized at 1 mg/ml in pre-warmed complete growth medium and stored until use at 20°C [29]. A 10 mM solution of the electron coupling agent menadione (Sigma) in acetone was prepared fresh and added to the XTT solution at a final concentration of 100 μ M immediately before use [30]. For measurement of metabolic activity of splenocytes, 50 μ l of pre-warmed XTT/menadione was added to each well after 48 h of cultivation. XTT cleavage as indicator of metabolic activity was assessed after incubation for 9 h at 37°C by absorbance measurement at 450 – 630 nm using a plate reader (Tecan, Groedig, Austria).

T cell line generation and stimulation

Ara h 2-specific oligoclonal CD4 + T cell lines (TCLs) were generated from peripheral blood mononuclear cells (PBMC) of peanut-allergic subjects as described [31] and stored in liquid nitrogen until use. Each TCL used in these experiments was specific for one of the five dominant T cell epitopes of Ara h 2 described by Prickett et al. [31] and did not react to any other peptide within Ara h 2. Proliferation assays were performed on 72-h triplicate cultures as described [31]. Antigens were untreated or r/a Ara h 2 or epitope peptides (all used at 10 μ g/mL) with untreated or r/a ovomucoid (10 μ g/mL) or culture medium alone as negative controls. Cells were pulsed with 3 H-thymidine (0.5 μ Ci/well) for the last 16 h and uptake measured as mean counts per minute (cpm) of triplicate cultures. Data are presented as a stimulation index (SI; cpm antigen-stimulated T cells/cpm unstimulated T cells). An additional 12 replica wells per antigen (without 3 H-thymidine) were pooled for RNA extraction (described below).

Spatial distribution of T cell epitopes in Ara h 2 structure [32] (PDB #3OB4) was visualized using the UCSF Chimera software (San Francisco, California; <http://www.cgl.ucsf.edu/chimera>) [33].

Gene expression analysis

Gene expression of human IL-5, IL-13 and IFN γ was analysed by Real-time PCR. The fold change as compared with unstimulated cells was calculated using the $\Delta\Delta$ CT method. A detailed description of RNA isolation, PCR protocol and primer sequences can be found in the material and methods section of the online supplement.

Statistical analysis

Data were statistically analysed using the (two-tailed) Mann–Whitney *U*-test using the software GraphPad Prism (version 5.0 for Windows, GraphPad Software, La Jolla, California). Differences with *P*-values ≤ 0.05 were considered to be significantly different. Thresholds: ***: *P*-value ≤ 0.001 ; **: *P*-value 0.001 – 0.01; *: *P*-value 0.01 – 0.05;

Results

Reduction and alkylation attenuates IgE binding and the cross-linking capacity of Ara h 2 *in vitro*

We hypothesized that the secondary structure (and associated conformational IgE epitopes) of Ara h 2 is an important factor for the allergenic and anaphylactic potential of the allergen. Application of an unfolded Ara h 2 variant could consequently decrease the risk of anaphylactic reactions during SIT. We aimed to test this hypothesis and prepared unfolded Ara h 2 by stabilizing the reduced state by alkylation (Fig. 1a,b) [25, 34]. We checked the purity of untreated and reduced and alkylated (r/a) Ara h 2 by SDS-PAGE, confirming the presence of the characteristic doublet of Ara h 2 isoforms in the absence of other protein bands (Fig. 1c). Analysis of the secondary structure by CD spectroscopy confirmed the unfolded state of r/a Ara h 2, represented by the single spectrum minimum close to 200 nm as compared with the CD spectrum typical for untreated Ara h 2 [35] and proteins of α -helical confirmation (Fig. 1d).

Alkylation stabilizes the reduced allergen by blocking sulphhydryl groups and might thereby decrease IgE binding to conformational epitopes and cysteine-containing linear epitopes. We compared the binding of IgE of human peanut-allergic patients with untreated and r/a Ara h 2. Ara h 2-specific IgE was detected in sera of 16 of 20 (16/20) diagnosed peanut-allergic patients (Table 1) by Western blot (Fig. 2a) and ELISA (Fig. 2b). Reduction/alkylation reduced the signal intensity by at least 25% in Western blot assays for 13/16 Ara h 2-reactive patients (Fig. 2a). A 20% or more decrease in IgE binding was also observed by ELISA for 11/16 of these patients (Fig. 2b). None of the investigated sera reacted against the control protein [alkylated human serum albumin (HSA)].

We next addressed the functional relevance of these observations and compared the capacity of untreated and r/a Ara h 2 to induce effector cell degranulation. Largely consistent with our Western blot and ELISA results, Ara h 2 stimulation induced degranulation of serum sensitized RBL-SX38 in 14/20 samples (Fig. 3). Alkylation reduced degranulation by 10% or more as compared with untreated Ara h 2 for 10 of these 14 samples (Fig. 3b).

Taken together, these *in vitro* experiments indicate reduced IgE binding and *in vitro* IgE cross-linking potential of r/a Ara h 2 as compared with the untreated form.

Alkylation marginally influences the response of human Ara h 2 specific T cell lines

The stimulation of T cells during SIT is considered to be important for successful therapy [10]. We therefore compared the response of human TCLs of known Ara h 2 epitope specificity [31] to either untreated or r/a Ara h 2, as well as to the respective T cell epitope peptide (Fig. 4a, Figure S1). Each TCL proliferated in response to its respective epitope peptide and to untreated Ara h 2. Stimulation with r/a Ara h 2 caused equal or greater proliferation as compared with untreated Ara h 2 in all TCLs except TCL-D1 (Fig. 4b and Figure S1). Neither incubation with untreated nor with r/a ovomucoid induced TCL proliferation (Figure S1).

Cytokines released by stimulated T cells initiate and shape immune responses. We analysed the expression of T_H1 [interferon (IFN) γ] and T_H2 [interleukin (IL) -5 and IL-13] cytokine mRNA in resting and stimulated TCLs. Consistent with proliferative responses, an increase in cytokine mRNA was observed for all TCLs following stimulation with specific antigen (peptide or either Ara h 2 preparation) compared with medium alone (Fig. 4c–e). The fold change in IL-5 mRNA (Fig. 4c) in TCLs stimulated with specific allergen was approximately 10-fold higher than in IL-13 (Fig. 4d) or IFN γ (Fig. 4e) mRNA. Stimulation of TCLs with the irrelevant negative control allergen ovomucoid (untreated or r/a) induced only marginal differences in cytokine mRNA levels as compared with untreated cells (Figure S1).

Stimulation with r/a Ara h 2 resulted in comparable or increased levels of mRNA for all three cytokines compared with untreated Ara h 2 for all TCLs except TCL-D1, which showed a marked decrease in IL-5 mRNA levels, consistent with the reduced proliferative response observed in Fig. 4b.

Alkylation decreases the anaphylactic potential of Ara h 2 *in vivo*

After assessment of IgE binding and degranulation capacity *in vitro*, we next aimed to verify whether alkylation influences the anaphylactic potential of Ara h 2 in an *in vivo* mouse model of systemic anaphylaxis. After confirming the successful induction of IgE antibodies specific for untreated Ara h 2 (Figure S2), we challenged the mice intravenously with untreated Ara h 2, r/a Ara h 2 or r/a ovomucoid. We measured body temperature as an immediate parameter of anaphylaxis and assessed the serum levels of mouse mast cell protease-1 (mMCP-1) 1 h after the challenge. Treatment with r/a Ara h 2 caused a reduced temperature drop as compared with application of the untreated form (Fig. 5a). These findings were reflected by lower mMCP-1 serum levels in mice challenged with r/a Ara h 2 (Fig. 5b). Of note, challenge with r/a ovomucoid neither affected body temperature nor mMCP-1 serum levels (Fig. 5a,b). The capacity of untreated and reduced/alkylated Ara h 2 to induce antibodies specific for the untreated allergen was head-to-head compared in additional immunization experiments. ELISA against untreated and reduced/alkylated Ara h 2 showed that the reduced variant retained statistically the same immunogenicity as the untreated variant and induced IgG1 antibodies, which were fully cross-reactive to the native, respectively, reduced and alkylated variant (Figure S3).

Alkylated Ara h 2 triggers increased metabolic activity and comparable cytokine release by spleen cells *ex vivo*

Next, we isolated spleen cells from the challenged animals and tested the metabolic response and cytokine release of splenocytes upon stimulation with untreated or r/a Ara h 2. Untreated and r/a Ara h 2 caused higher metabolic activity than r/a ovomucoid (Fig. 6a). Among the proteins, r/a Ara h 2 caused the most profound stimulation. Of note, we did not observe a difference in the pattern of splenocyte activity between cells isolated from mice that had been challenged with untreated Ara h 2 (left column) and those that had been challenged with r/a Ara h 2 (right column). The Ara h 2-induced metabolic activity was specific and dependent on the foregoing sensitization of the animals, as splenocytes from naïve mice did not respond to antigen stimulation (Figure S4).

We analysed the splenocyte release of IFN γ , IL-5 and IL-13 and found similar production of IL-5 (Fig. 6b) and IL-13 (Fig. 6c) upon stimulation with untreated and r/a Ara h 2 irrespective of the previous challenge of animals. Although not significant, r/a Ara h 2 seemed to induce less IFN γ secretion (Fig. 6d) than the untreated form. Splenocytes of naïve mice did not produce any of the tested cytokines upon antigen stimulation (Figure S4).

Discussion

Specific immunotherapy is currently the only form of disease-modifying treatment available for allergic individuals [12]. However, due to the high risk of systemic reactions [19], SIT is not clinically applicable for the treatment of food allergy. The use of hypoallergenic variants of proteins or extracts might be a promising alternative approach. In this study, we investigated allergenicity and anaphylactic potential of an unfolded food allergen and addressed its suitability for SIT.

We produced a stably linearized variant of the major peanut allergen Ara h 2 by reduction and alkylation (r/a Ara h 2; Fig. 1d). R/a Ara h 2 exhibited reduced binding to IgE of peanut-allergic patients as compared to untreated Ara h 2 (Fig. 2). In line with decreased IgE binding, r/a Ara h 2 also caused reduced effector cell degranulation (Fig. 3), suggesting markedly decreased anaphylactic potential. It can be anticipated that the r/a changes may affect the ‘valency’ of the antigen rather than the affinity of those antibodies [36]. In line with that assumption, we could demonstrate that the mediator release for both allergen variants reached – concentration dependent – a maximum response at 1000 ng/ml and decreased again at higher concentrations (data not shown).

While an attenuated IgE binding and degranulation capacity would be favourable for SIT application, the T cell stimulatory potential of the applied allergen variant should be retained [12]. We stimulated Ara h 2-specific human T cell lines with untreated and r/a Ara h 2 and observed that r/a Ara h 2 had a similar, if not increased stimulatory potential compared to untreated allergen for seven out of the eight TCLs tested (Fig. 4b). The decreased response observed for TCL-D1 to r/a Ara h 2 might be explained by its specific epitope sequence, which contains two cysteine residues (as compared with only one cysteine in the epitopes of the other tested TCLs) (Fig. 4a). We showed previously that replacing the cysteine residues with serine residues in this epitope did not affect responses of this line [31], but it may be that the presence of chemically modified cysteines (Fig. 1a) in this epitope might cause altered epitope processing or interfere with its binding to HLA molecules or the T cell receptor.

T cells are important initiators and enhancers of immune responses. We analysed mRNA levels of representative T_H1 and T_H2 cytokines in stimulated human TCLs. R/a Ara h 2 tended to induce similar or higher levels of IL-5 (Fig. 4c), IL-13 (Fig. 4d) and IFN γ (Fig. 4e) mRNA as compared with the untreated form. We conclude from these experiments that r/a Ara h 2 has similar, if not higher T cell stimulatory potential as compared with the untreated allergen.

One major problem of food allergen SIT is the high risk of systemic anaphylaxis upon allergen injection [19]. We therefore compared the anaphylactic potential of untreated vs. r/a Ara h 2 in mice sensitized with untreated Ara h 2. Intravenous challenge with untreated Ara h 2 caused severe anaphylactic reactions reflected by a significant temperature drop (Fig. 5a) and increased serum levels of the mast cell protease mMCP-1 (Fig. 5b). In contrast, challenge with r/a Ara h 2 caused only low-grade anaphylactic symptoms (Fig. 5a, b). While work by Toda et al. reported that reduction and alkylation rendered hypoimmunogenicity besides hypoallergenicity to the major peach allergen Pru p 3 [26], we demonstrate here that r/a Ara h 2 retains its full immunogenicity and thus capacity to induce IgG (Figure S3).

We isolated spleen cells from Ara h 2-sensitized and challenged mice and investigated their response to allergen exposure. Spleen cells stimulated with r/a Ara h 2 had similar metabolic activity as compared to untreated Ara h 2 (Fig. 6a). The analogous release of T_H2 cytokines (IL-5, IL-13) and a T_H1 cytokine (IFN γ) (Fig. 6b–d) by cells stimulated with either Ara h 2 variant was in agreement with the mRNA data obtained with Ara h 2-specific human T cell lines (Fig. 4c–e). Splenocytes from naïve mice did not respond to allergen stimulation, proving the specificity of the response (Figure S3). These *in vivo* and *ex vivo* experiments provide evidence that r/a Ara h 2 has decreased anaphylactic potential while retaining a T cell stimulatory capacity comparable to untreated Ara h 2.

Successful SIT is associated with the induction of a T_H1 bias or regulatory T cells [12]. As r/a Ara h 2 exposure alone does not appear to induce the desired shift towards production of T_H1 cytokines, the co-application of T_H1 adjuvants like CpG-rich oligonucleotides (CpG-ODNs) during therapy might be necessary [37, 38].

In conclusion, our experiments provide evidence that alkylation has the potential to decrease the allergenicity and anaphylactic potential of the major peanut allergen Ara h 2. However, it should be kept in mind that peanuts contain additional important allergens, including Ara h 1 or Ara h 6 [39–41]. The concept here presented for Ara h 2 should therefore be tested next for additional peanut allergens and, finally, for whole peanut extract. Furthermore, it will be important to investigate the potential benefit of r/a allergen SIT in a therapeutic mouse model of established peanut allergy. These experiments should reveal the suitability of alkylated allergens for SIT of food allergies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Alkylated Ara h 2 is stably unfolded. (a, b) Alkylation of sulfhydryl groups of cysteine side chains: (a) Under reducing conditions at basic pH, the ethyl group of iodoethanol attaches irreversibly to free sulfhydryl groups of cysteine contained in peptides, thereby masking the reactive sulfhydryl group and avoiding re-establishment of disulphide bridges under oxidizing conditions. (b) The unfolded state (lower scheme) of a protein can be stabilized by alkylation as schematically indicated. 'S' represents a sulphhydryl group of cysteine, alkylated sulphhydryl groups are indicated in red. (c) Purity of Ara h 2. Ara h 2 was purified

from peanuts. Untreated (lane 2) and reduced/alkylated (r/a) Ara h 2 (lane 3) were separated by SDS–PAGE and stained with Coomassie Brilliant blue. Lane 1: protein marker; numbers left of lane 1 indicate the molecular weight (in kDa) of the respective marker band. (d) r/a Ara h 2 is unfolded. The secondary structure of untreated (untr.) and r/a Ara h 2 was analysed by circular dichroism spectroscopy.

Fig. 2.

Alkylation affects IgE binding to Ara h 2. (a) Western blot: Binding of serum IgE of human allergic patients to identical amounts of untreated (upper row) and reduced/alkylated (r/a) (middle row) Ara h 2 was analysed by Western blot. The band intensity was analysed by densitometry and the percentage difference in IgE antibody binding induced by alkylation as compared to untreated Ara h 2 is presented (lower row). NA = serum from non-allergic donor; B = Buffer control. (b) ELISA: Binding of serum IgE from peanut-allergic subjects to untreated (untr.) and r/a Ara h 2 as well as r/a human serum albumin (HSA, control protein)

was analysed by ELISA. The absorbance values at 405–490 nm are displayed (upper panel). The lower panel shows the calculated, alkylation-induced difference relative to untreated Ara h 2.

Fig. 3.

Alkylation reduces the ability of Ara h 2 to induce effector cell degranulation. Effector cell degranulation: RBL-SX38 cells were sensitized with serum from peanut-allergic subjects and then stimulated with untreated or reduced/alkylated (r/a) Ara h 2. (a) The percentage of released β -hexosaminidase in relation to total β -hexosaminidase content was calculated. (b) The alkylation-induced difference in β -hexosaminidase release relative to stimulation with untreated Ara h 2 is shown. NA: serum from non-allergic donor.

Fig. 4.

The response of human Ara h 2-specific T cells to untreated and r/a Ara h 2 is comparable. (a) T cell line (TCL) core epitopes: The amino acid (AA) sequences of Ara h 2 core T cell epitopes (equivalent to the peptides used for TCL stimulation) and their respective position within the complete Ara h 2 AA sequence is shown. The sequences are presented in single letter code and cysteines, being potential alkylation targets, are labelled in red. The spatial distribution of core epitopes is visualized in Figure S1. (b) Human T cell line proliferation: Ara h 2-specific human T cell lines (TCLs) were stimulated with untreated (untr.) or reduced/alkylated (r/a) Ara h 2, or the respective core epitope peptide. Proliferation was measured by ^3H -labelled thymidine uptake and data were expressed as stimulation indices (SI: fold increase in thymidine uptake by stimulated cells over cells cultured in plain

medium; please see also the ^3H -thymidine uptake levels in Figure S1). The letters under the TCL name indicate the core peptide specificity as shown in (a). *t*-test (two-tailed) statistical analysis between samples stimulated by untreated and reduced/alkylated Ara h 2; samples without asterisk were not significantly different; (c–e) Human TCL cytokine mRNA expression: TCLs were stimulated as described in (b) and mRNA was isolated and processed. Expression levels of genes coding for (c) IL-5, (d) IL-13 and (e) IFN γ were analysed by real-time PCR. The expression fold change in mRNA levels for stimulated as compared to unstimulated cells was calculated.

Fig. 5.

Alkylated Ara h 2 has attenuated anaphylactic potential in sensitized mice. (a) Body temperature kinetics: BALB/c mice were sensitized intraperitoneally with untreated Ara h 2 and challenged intravenously with untreated ($n = 7$) or reduced/alkylated (r/a) ($n = 7$) Ara h 2 or r/a ovomucoid (Ovo) ($n = 6$) or saline ($n = 8$). Body temperature was measured immediately before (= time point 0) and after challenge as indicated. Mann–Whitney U -test: untr. Ara h 2 vs. r/a Ara h 2: *: P -value 0.01–0.05; r/a Ara h 2 vs. r/a Ovo: +: P -value 0.01–0.05; r/a Ara h 2 vs. Saline: °: P -value 0.01–0.05; (b) mMCP-1 ELISA: mMCP-1 serum levels 1 h after challenge with untreated ($n = 7$) or r/a ($n = 7$) Ara h 2 or r/a Ovo ($n = 6$) or saline ($n = 8$) were analysed by ELISA. Mann–Whitney U -test; ***: P -value 0.001; **: P -value 0.001 – 0.01; *: P -value 0.01 – 0.05; ns = not significant.

Fig. 6.

Alkylation influences the response of allergen-primed mouse splenocytes *ex vivo*. Spleen cells of mice sensitized with untreated Ara h 2 and intravenously challenged with either untreated (untr.; left column; $n = 7$) or reduced/alkylated (r/a; right column, $n = 7$) Ara h 2 were isolated and stimulated *ex vivo* with plain culture medium, untreated or r/a Ara h 2, r/a ovomucoid (Ovo) or concanavalin A (Con A). (a) Activity assay: The metabolic activity of allergen-stimulated splenocytes was assessed using an XTT assay and compared to splenocytes stimulated with plain culture medium (= 100%). (b–d) Cytokine release:

Supernatant of spleen cells was collected after 72 h of cultivation. Released (b) IL-4, (c) IL-13 and (d) IFN γ were analysed by ELISA. Mann–Whitney *U*-test; ***: *P*-value 0.001; **: *P*-value 0.001–0.01; *: *P*-value 0.01–0.05; ns = not significant.

Table 1
Clinical characteristics of patients and serum reactivity to peanuts

#	code	Age years	sex (f/m)	Allergy grade [*]	peanut-specific IgE [kU/L] [†]
1	91	5	M	1/3	58.4
2	106	10	F	1/3	> 100
3	114	2	M	1	4.59
4	119	3	F	1/3	31.2
5	122	7	M	1/2/3	7.4
6	157	6	F	0	87.4
7	158	2	F	1/3	3.34
8	163	18	M	1/2	69.8
9	273	4	M	1/3	> 100
10	290	8	F	0/1	29
11	295	3,8	F	1	49.4
12	301	8	F	1	> 100
13	320	5,9	M	1/3	19.5
14	324	7,5	M	1/2/3	79
15	394	15	M	0/1/3	3.89
16	421	11	F	0/1/3	> 100
17	430	3	M	1	2.38
18	445	5,5	F	1/2/3	> 100
19	567	14,6	M	0/1/3	11.8
20	648	6	M	0/1/3	> 100

Sera of 20 peanut-allergic patients were applied for the *in vitro* experiments (Fig. 1–3) in this study. Age, gender, symptoms and peanut-specific IgE levels are presented. Symptoms are described according to the Muller score of allergic symptoms [42]: 0: symptoms of the oral cavity, 1: symptoms of the skin and mucous membranes, 2: gastrointestinal symptoms, 3: respiratory symptoms, 4: cardiovascular symptoms; serum levels of peanut-specific IgE antibodies were determined using ImmunoCAP (Phadia AB, Vienna, Austria); M, male; F, female.

^{*} Muller score of allergic symptoms: 0: symptoms of the oral cavity, 1: symptoms of the skin and mucous membranes, 2: gastrointestinal symptoms, 3: respiratory symptoms, 4: cardiovascular symptoms.

[†] determined using ImmunoCAP (Phadia AB, Vienna, Austria).