Allergenic characteristics of a modified peanut allergen

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Attempts to treat peanut allergy using traditional methods of allergen desensitization are accompanied by a high risk of anaphylaxis. The aim of this study was to determine if modifications to the IgE-binding epitopes of a major peanut allergen would result in a safer immunotherapeutic agent for the treatment of peanut-allergic patients. IgE-binding epitopes on the Ara h 2 allergen were modified, and modified Ara h 2 (mAra h 2) protein was produced. Wild-type (wAra h 2) and mAra h 2 proteins were analyzed for their ability to interact with T-cells, their ability to bind IgE, and their ability to release mediators from a passively sensitized RBL-2H3 cell line. Multiple T-cell epitopes were identified on the major peanut allergen, Ara h 2. Ara h 2 amino acid regions 11 – 35, 86 – 125, and 121 – 155 contained the majority of peptides that interact with T-cells from most patients. The wAra h 2 and mAra h 2 proteins stimulated proliferation of T-cells from peanut-allergic patients to similar levels. In contrast, the mAra h 2 protein exhibited greatly reduced IgE-binding capacity compared to the wild-type allergen. In addition, the modified allergen released significantly lower amounts of β-hexosaminidase, a marker for IgE-mediated RBL-2H3 degranulation, compared to the wild-type allergen.

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1 Introduction

In North America and Europe a large portion of the population is affected by allergic reactions to common environmental proteins, making atopic disorders among the most prevalent diseases in the industrialized nations [1]. In the most severe cases, exposure to an allergen in sensitive individuals can produce a life-threatening anaphylactic response. For example, allergy to food proteins is the most common single cause of anaphylaxis outside hospitals in the United States [2 – 4].

Documented food hypersensitivity reactions affect approximately 6 – 8% of young children and 3 – 5% of adults [5]. Peanuts, milk, and eggs account for the majority of food allergic reactions in children, while peanuts, tree nuts, and shellfish are responsible for the majority of reactions in adults [6]. Most children will outgrow allergies to foods such as milk, eggs, wheat, and soybean. Unfortunately, allergy to peanuts will typically persist into adulthood, lasting the entire lifetime of the individual. However, peanut allergy in a small proportion of young children has been shown to resolve with age [7]. It is estimated that about 0.6% of the United States population is allergic to peanuts [8].

Despite the prevalence of peanut hypersensitivity in the population, and an increasing number of deaths each year from peanut-induced anaphylaxis, the only treatment available for peanut-allergic patients is strict avoidance of peanut allergens [9]. Recently, the food industry has started using peanuts as a supplemental protein source in a wide variety of processed food [10]. This inclusion of potential hidden peanut allergens makes accidental consumption almost unavoidable for peanut-sensitive individuals. In fact, in a retrospective study of peanut-induced clinical symptoms, 50% of allergic individuals suffered a peanut-induced hypersensitivity reaction during a 2-year period [11].

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Certain allergic sensitivities, such as to inhaled pollens and insect sting venom, have been successfully treated by traditional, allergen-specific desensitization. This immunotherapeutic regimen involves the subcutaneous injection of progressively higher doses of allergen to produce immunologic tolerance. Immunologic tolerance is induced by redirecting the T-cell immune response from a Th2- to a Th1-type response [12, 13]. However, the treatment of food allergy by injecting allergen extracts has failed due to the limited efficacy and unwanted side-effects, including anaphylaxis and death [14]. Therefore, it is necessary to explore strategies designed to lower the ability of food allergens to elicit dangerous allergic responses yet retain their ability to interact with T-cells and redirect the immune system to a tolerant state [15].

The major peanut allergen, Ara h 2, is recognized by serum IgE from >90% of peanut-sensitive patients [16]. Ara h 2 is a 17 kDa glycoprotein belonging to the 2S seed storage protein family. Ten linear IgE-binding epitopes have been located in the amino acid sequence of recombinant Ara h 2, using synthetic peptides that represent overlapping fragments of the entire protein and a serum IgE pool from peanut-allergic patients [17]. Individual amino acids were substituted in linear peptides representing the Ara h 2 IgE-binding epitopes that were identified as amino acids critical for IgE binding [17].

In this study, we utilized information generated from mutagenesis of Ara h 2 peptides to develop a hypoallergenic variant of the intact Ara h 2 protein that exhibited properties favorable for allergen immunotherapy in *in vitro* assays. These properties included the need for larger amounts of the modified allergen in an ELISA-based inhibition assay for serum IgE from a pool of peanut-allergic patients, reduced binding of peanut-specific IgE from individual patients to the modified protein, and the retained ability of the modified protein to stimulate the proliferation of T-cells from peanut-allergic patients. In an effort to test the *in vivo* efficacy of this modified allergen, the release of β-hexosaminidase from an RBL-2H3 cell line passively sensitized with IgE from peanut-allergic patients was assessed after challenge with either the wild-type allergen or the modified Ara h 2 (mAra h 2) protein. Challenge with the mAra h 2 proteins caused the release of significantly lower amounts of cell mediators when compared with the wild-type allergen.

2 Materials and methods

2.1 Serum IgE

Sera from patients with documented peanut hypersensitivity were used to probe native and recombinant peanut proteins in order to examine IgE-binding reactivity. Each patient had a positive immediate prick skin test to peanut and either a positive, double-blind, placebo-controlled, food challenge (DBPCFC) or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension). A serum pool, consisting of equal aliquots of serum IgE from each of 20 peanut-allergic patients or individual serum samples from patients comprising the serum pool, was used in immunoblot analysis experiments to determine the IgE-binding characteristics of the population. Details outlining the challenge procedure and collection of sera containing peanut-specific IgE have been discussed previously [15]. All studies were approved by the Human Use Advisory Committee at the University of Arkansas for Medical Sciences and Mount Sinai School of Medicine.

2.2 Site-directed mutagenesis of the Ara h 2 gene by the PCR

The 5′ untranslated region (UTR) and the signal sequence of the protein were removed from the peanut cDNA clone. In addition, the stop codon and the UTR were removed from the 3′ end. The Ara h 2 cDNA was then ligated into a pET24 vector so that a T7 polymerase epitope tag was fused in frame to the 5′ end and a polyhistidine tag was fused to the 3′ end. Codons corresponding to the amino acids critical for IgE binding were replaced by alanine codons using PCR amplification with the *Pfu* DNA polymerase from Stratagene (LaJolla, CA) or *Accuzyme* from ISC (Kaysville, UT) and epitope-specific primers. The wild-type Ara h 2 (wAra h 2) insert in a BLUESCRIPT (SK-) plasmid was used as a template for mutagenesis. The conditions for all amplification reactions were as follows. The mixture was denatured at 94°C for 30 s, annealed at 60°C for 30 s, and extended at 72°C for 1 min for 25 cycles. The reactions were extended for 7 min at 72°C. Constructs containing the Ara h 2 gene with mutations in all ten IgE-binding epitopes were used for the expression of fully modified recombinant Ara h 2 protein.

2.3 Expression and purification of recombinant Ara h 2 proteins

Expression constructs containing wAra h 2 or mAra h 2 sequences in pET24 vectors were transformed into *Escherichia coli* strain BL21(DE3) cells. Exponentially growing cells were induced with 1 mM IPTG and grown for another 3 h. For SDS-PAGE/immunoblot analysis, preinduced and induced cells were harvested, diluted in SDS-PAGE sample buffer, and electrophoresed on 12% gradient polyacrylamide gels. Proteins separated by SDS-PAGE were transferred onto an NC membrane and probed with antibodies

directed against the T7 epitope tag to detect the recombinant protein.

All recombinant Ara h 2 proteins were purified from bacterial lysates under denaturing conditions using a His-Bind Purification Kit (Novagen, Madison, WI, USA). Briefly, cell extracts were resuspended in 4 mL of cold Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, and 6 M urea), sonicated for 3×30 s intervals by a Model 300 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA), and incubated on ice for 1 h. The lysate was centrifuged at $12\,000 \times g$ for 45 min in a Beckman J2-21 centrifuge (Beckman Instruments, Fullerton, CA, USA) to remove cellular debris. The supernatant was clarified by passing it through a 0.45-µm membrane using a syringe-end filter. A 5 mL His-Bind Quick Column was packed with His-Bind metal chelation resin, washed with deionized H_2O, and charged with Charge Buffer (50 mM $NiSO_4$). The column was equilibrated with Binding Buffer and 2 vol. of supernatant allowed to flow through the column. The column was washed with 10 vol. of Binding Buffer and 6 vol. of Wash Buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 6 M urea). Elution was achieved with 5 vol. of Elution Buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 6 M urea).

Fractions containing recombinant proteins were determined by SDS-PAGE/immunoblot analysis and placed in Snakeskin™ pleated dialysis tubing (Pierce Chemical, Rockford, IL, USA) with a molecular weight cutoff of 3500 Da and dialyzed against 1 M urea in PBS. After 16 h, dialysis tubing was moved to a new solution for overnight dialysis against 0.1 M urea in PBS. A third dialysis against PBS was performed prior to the use of recombinant protein for experiments. All purified recombinant proteins were stored in PBS, or in a solution of 0.1 M urea in PBS, at 4°C.

2.4 SDS-PAGE, Western blots, and IgE-binding assay

Purified recombinant proteins and bacterial extracts were analyzed by SDS-PAGE using precast 12% SDS-PAGE gels (Novex, San Diego, CA, USA). Proteins were visualized by either CBB staining or by using Gelcode Blue Stain Reagent according to manufacturer's protocol (Pierce Chemical). For immunoblot analysis, proteins were electroblotted onto NC. After transfer, blots were blocked using a solution containing Tris/NaCl and 3% BSA. All blots were incubated with a serum pool from patients with documented peanut hypersensitivity or individual serum diluted (1 : 5) in a solution containing Tris/NaCl and 1% BSA for 16 h at 4°C. An ^{125}I-labeled antihuman IgE antibody and autoradiography were used to detect the amount of IgE binding to each of the proteins. In the competition/inhibition experiments, varying concentrations of wild-type or modified allergen were mixed with pooled serum IgE that was diluted (1 : 10) prior to being used in the ELISA inhibition assay. Primary antibody was detected with an HRP-coupled anti-IgE antibody (Kirkegaard Perry Labs, Gaithersburg, MD).

2.5 T-cell proliferation assay

Four to six milliliters of heparinized blood from peanut-allergic patients were added to sterile tubes containing 3 mL of Fico/Lite (density 1.077; Atlanta Biologicals, Atlanta, GA, USA). Separation was achieved by spinning the tubes at 1800 rpm for 30 min in a Sorvall RT6000R Centrifuge (Sorvall Products, Newton, CN, USA). After separation, the top layer of plasma and the monocytic layer containing the lymphocytes were placed into clean, sterile tubes and spun at 1800 rpm for 10 min. The resulting plasma supernatant was saved for future use and the cell pellet lifted with PBS. Following resuspension, the tube was centrifuged at 1800 rpm for 10 min. After this final wash, 3.8 mL RPMI (10% FBS) (Atlanta Biologicals) was added to each tube. Cell viability was determined by adding 20 µL of Trypan Blue to 20 µL cells and counting the number of dead cells *per* 100 cells. The cell number was adjusted to 2×10^6 cells/mL. Lymphoblast assays were setup in triplicate with 10 µg of protein added to 100 µL of cells in a sterile round-bottom 96-well plate. Wells containing only media served as background controls. Plates were incubated at 37°C for 3 days. On day 3, cells were tagged with 50 µL (1 µCi) of [^{3}H]-thymidine and incubated for 6 h at 37°C. After incubation, cells were harvested onto glass filter paper and dried overnight. The amount of incorporated label was determined with a Beta counter according to manufacturer. The stimulation index (SI) was calculated by dividing the mean cpm incorporation of the triplicate experimental values by the mean cpm in cell cultures where only media was added.

2.6 Mediator release from a passively sensitized RBL-2H3 cell line

The rat basophil leukemia cell subline RBL-2H3 was transfected with the α chain of the human Fc_ε RI receptor in order to efficiently bind human IgE [18]. RBL-2H3 cells were cultured in Eagle's MEM with 10% FCS, 0.1% geneticin sulfate, harvested in the stationary phase, and transferred to 96-well microtiter plates (1.5×10^5 cells *per* well). Cells were passively sensitized by incubation with human serum IgE for 18 h at 37°C and 5% CO_2 prior to performing the mediator release assay [19]. Dilutions of sera from peanut-allergic patients were optimized for maximum mediator release by preliminary titrations in Eagle's MEM buffer. After sensitization, the adherent cell layer was washed three

times with Tyrode's buffer and incubated with 100 µL of serial dilutions of the cross-linking agents (allergens or extracts) in Tyrode's buffer containing 50% D_2O [20] for 1 h at 37°C. For convenience, β-hexosaminidase was measured. β-hexosaminidase is released at the same rate as histamine [20, 21] and, therefore, is a suitable marker for IgE cross-linking mediated degranulation. To determine β-hexosaminidase activity, 30 µL of the supernatant was transferred into a new microtiter plate and incubated with 50 µL of the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (1.3 mg/mL in 0.1 M phosphate, 0.05 M citrate, pH 4.5) for 1 h at 37°C. After addition of 100 µL of 0.2 M glycine, pH 10.7, the absorbance was read at 405 nm (reference: 620 nm). As a control for each microtiter plate, cells were sensitized with human myeloma IgE (hu IgE) (Biogenesis, Poole, UK; 1:5000) and stimulated with goat antihuman IgE (Nordic, Tilburg, NL, USA: 1:1000). Spontaneous release was determined by omitting the cross-linking agents, and the total enzyme content was measured by lysing the cells with 1% Triton X-100. Allergen-specific release was calculated as percent of β-hexosaminidase release after correction for spontaneous release.

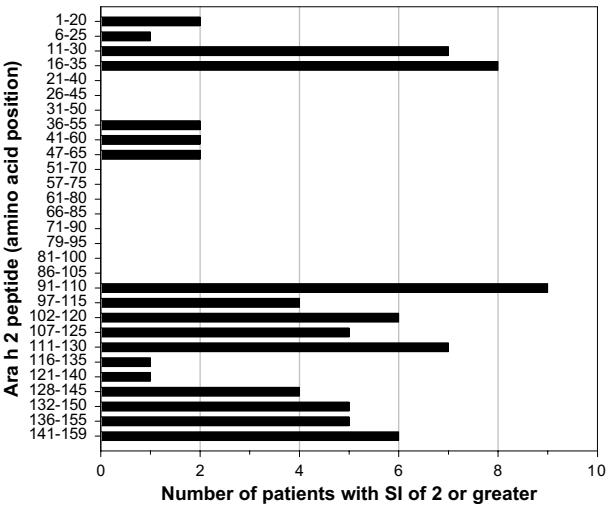


Figure 1. Ara h 2 peptides that stimulate T-cell proliferation. Individual synthetic overlapping peptides, 20 amino acids in length overlapped by 15 from the preceding peptide, were produced for the entire Ara h 2 protein and used in T-cell proliferation assays from PBMCs collected from 14 peanut-sensitive patients. Histograms indicate the number of patients that responded to individual peptides with an SI of 2 or greater.

3 Results

3.1 Mapping T-cell epitopes on the Ara h 2-protein sequence

In order to determine those regions of the Ara h 2 protein that interact with T-cells, overlapping synthetic peptides were synthesized. Twenty-nine molecules, representing the entire native Ara h 2 protein in 20 amino acid long peptides offset from each other by five amino acids, were utilized in T-cell proliferation assays to identify those regions representing T-cell epitopes. PBMCs collected from 14 different peanut-sensitive patients were assayed for their ability to proliferate in the presence of each of the Ara h 2 peptides. The data from these experiments are presented in Fig. 1. The data indicate that, as expected, there are multiple T-cell epitopes located in the Ara h 2 protein and that these epitopes vary from patient to patient. However, Ara h 2 amino acid regions 11–35, 86–125, and 121–155 appear to contain the majority of peptides that interact with T-cells from most patients.

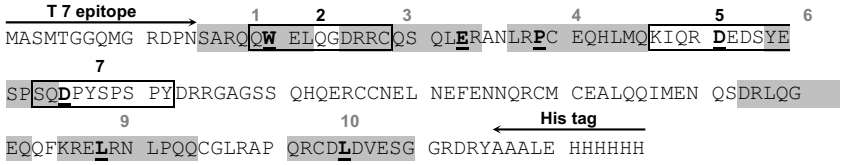
3.2 Construction of an mAra h 2 cDNA clone and IgE-binding properties of an mAra h 2 protein

We have previously reported the location of ten linear IgE-binding epitopes in the primary amino acid sequence of the major peanut allergen, Ara h 2. In addition, amino acids within each linear epitope were identified that, when changed to alanine, resulted in loss of IgE binding [17]. A modified version of the Ara h 2 protein was produced by site-directed mutagenesis of the cDNA within each of these epitopes, resulting in an Ara h 2 protein different from the wild-type at amino acid positions 20, 33, 39, 51, 58, 64, 117, 127, and 144 (Fig. 2A). A single change at position 20 greatly diminished IgE binding to peptides containing epitopes 1 and 2.

In order to assess the IgE-binding capacity of the mAra h 2, immunoblot assays and ELISA inhibition assays were performed using pooled serum IgE from documented peanut-sensitive patients. In the immunoblot assay, equal amounts of the wAra h 2 and mAra h 2 proteins (2 µg each) were electrophoresed on 12% SDS-PAGE gels, transferred to NC, and then incubated with pooled serum IgE from peanut-sensitive patients. As shown in Fig. 2B, the amount of IgE bound to the mAra h 2 protein was dramatically reduced when compared to the IgE bound to the wAra h 2 protein.

The ability of the mAra h 2 protein to inhibit IgE binding to the wAra h 2 protein was determined by an ELISA inhibition assay using the same pooled serum IgE as described for the immunoblot assay. Different concentrations of the wAra h 2 or mAra h 2 proteins were incubated in solution with the pooled serum IgE prior to adding the mixture to a microtiter plate previously adsorbed with 50 ng of wAra h 2 protein. An HRP coupled antihuman IgE antibody was used to determine the amount of IgE binding to the wAra h 2 protein. The results are shown in Fig. 2C. To obtain 50% inhi-

A



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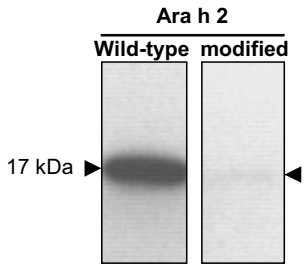

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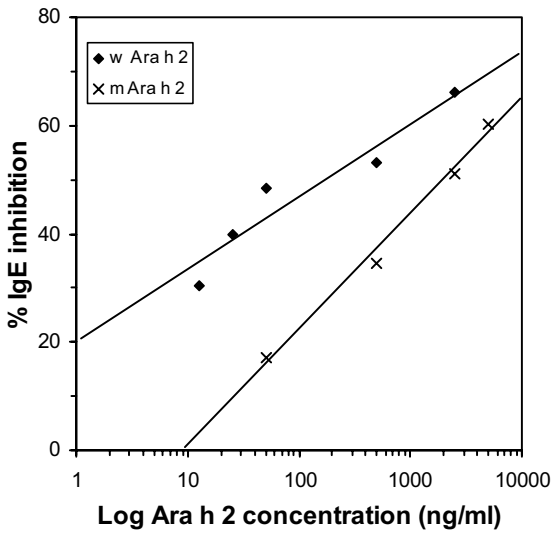

Figure 2. Panel A: Amino acid sequence of Ara h 2. Primary sequence of Ara h 2 is shown as the one letter amino acid code. Boxes (gray and clear) represent those regions previously shown to be IgE-binding epitopes [17]. Bold, underlined amino acids indicate residues that were changed to alanine by site-directed mutagenesis of the Ara h 2 cDNA. A T7 tag sequence (aa 1 – 14) and a HIS tag sequence (aa 156 – 166) were added to the Ara h 2 protein. Panel B: Immunoblot analysis of the wAra h 2 and mAra h 2 proteins. Recombinant wAra h 2 and mAra h 2 cDNA pET24 constructs were induced to express in *E. coli* strain BL21(DE3) cells. Recombinant proteins were purified by affinity chromatography over an Ni^{+} column and 2 μg of each protein electrophoresed on 12% SDS-PAGE gels. Proteins were transferred to an immobilon membrane and incubated with a pool of serum IgE from 15 peanut-sensitive patients. IgE binding was detected by ^{125}I-antihuman IgE. Panel C: Increased amounts of mAra h 2 protein are required to inhibit IgE binding to the wAra h 2 protein. wAra h 2 protein was allowed to adhere to the wells of microtiter dishes. Pooled sera containing allergen-specific IgE from peanut-sensitive patients were incubated with increasing concentrations of either the wAra h 2 (υ) or the mAra h 2 protein (6) and then placed in the microtiter wells. Relative amounts of IgE binding to the wAra h 2 protein was then determined by an antihuman IgE antibody coupled to HRP.

bition of IgE binding to the wAra h 2 protein required greater than a log-fold increase in the mAra h 2 protein when compared to a similar level of inhibition obtained using the wild-type protein.

3.3 mAra h 2 proteins bind reduced amounts of serum IgE from individual peanut-sensitive patients

The results reported in Section 3.2 indicate that it is possible to dramatically reduce the amount of IgE binding to the mAra h 2 proteins when assayed in the context of a pool of peanut-sensitive patients' sera. However, it is important to determine whether peanut-sensitive individuals exhibit reduced IgE binding to the mAra h 2 protein if it is to be used as a general immunotherapeutic agent for peanut allergy. Therefore, the ability of the mAra h 2 protein to bind IgE from individual patients was determined by immunoblot analysis. Serum IgE from 52 different peanut-sensitive patients were incubated with immunoblot strips containing equal amounts (2 μg) of the wAra h 2 or mAra h 2 proteins, and the amount of IgE bound to each strip was determined as described above. Individual patient serum

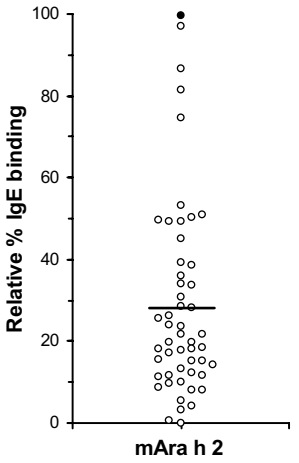

Figure 3. mAra h 2 protein binds less IgE from peanut-sensitive patient sera than the wAra h 2 protein. Recombinant wAra h 2 and mAra h 2 cDNA pET24 constructs were induced to express in *E. coli* strain BL21(DE3) cells. Recombinant proteins were purified by affinity chromatography over an Ni^{+} column and 2 μg of each protein electrophoresed on 12% SDS-PAGE gels. Proteins were transferred to an immobilon membrane and incubated with serum IgE from individual patients. IgE binding was detected by ^{125}I-antihuman IgE, quantitated by densitometry, and expressed as % IgE binding to the wAra h 2 protein.

IgE binding to the mAra h 2 protein was expressed as a percentage of IgE binding to the wAra h 2 protein (Fig. 3). Mutation of all ten IgE-binding epitopes in the mAra h 2 protein led to a significant decrease (>50%) of IgE binding by 46/52 patient sera tested. The mean of the amount of IgE binding to the mAra h 2 protein for this population of peanut-sensitive patients was 29.6% of that bound to the wild-type protein.

3.4 The mAra h 2 protein retains the ability to interact with T-cells and stimulate PBMC proliferation

For the mAra h 2 protein to be useful as a desensitizing agent for peanut immunotherapy, it must retain its ability to interact with T-cells. Since many of the Ara h 2 regions identified as T-cell epitopes also contained IgE-binding epitopes, it was of interest to determine whether any of the amino acid changes made to the mAra h 2 protein prevented this protein from interacting with T-cells from peanut-sensitive patients. Proliferation assays were performed on PBMCs collected from peanut-sensitive patients using either the wAra h 2 or the mAra h 2 protein, and the results are shown in Fig. 4. The T-cell SI for wAra h 2 was arbitrarily set at "1" for each patient. The T-cell SI obtained with the mAra h 2 protein was expressed as a fraction relative to that obtained for the wild-type allergen. The relative SI for

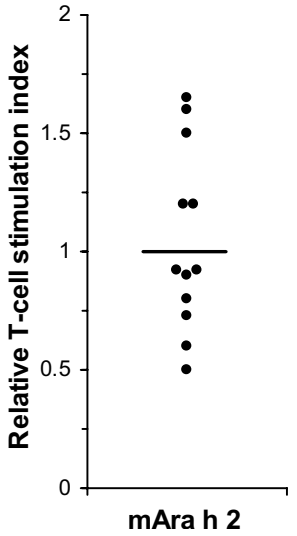

Figure 4. mAra h 2 stimulates T-cells to proliferate to the same extent as the wAra h 2 protein. PBMCs from peanut-allergic patients were stimulated to proliferate with either the wAra h 2 protein or the mAra h 2 protein. Blast assays were setup in triplicate with 10 μg of protein added to 100 μL of cells in a sterile round-bottom 96-well plate. Wells containing only media served as background controls. An SI was calculated by dividing the mean cpm incorporation of the triplicate experimental values by the mean cpm added to the media. A ratio of the SI from cells incubated with wAra h 2 and mAra h 2 was then determined for each patient tested and expressed as the relative T-cell SI. Each point represents this ratio for a single peanut-allergic patient.

the mAra h 2 protein was 1.12 ± 0.20 for all patients tested. These results suggest that the amino acid changes made to the mAra h 2 do not prevent this protein from stimulating T-cells to proliferate to a similar degree as the wild-type protein.

3.5 The mAra h 2 protein releases significantly lower amounts of mediators from a passively sensitized RBL-2H3 cell line

In an effort to determine whether the mAra h 2 protein would elicit lower amounts of allergic mediators from effector cells, an RBL-2H3 cell line was passively sensitized with serum IgE from peanut-allergic patients. These cells were then challenged with varying amounts of total peanut extract, Ara h 2 protein isolated from peanuts, wAra h 2, mAra h 2, soybean extracts, or pea extracts, and the relative amounts of β-hexosaminidase that were released from each was monitored (Fig. 5). In these experiments, the amount of β-hexosaminidase released after lysis of the cells with Triton X-100 was set at 100%, and all other values were expressed as a percentage of this value. For each sera tested, the mAra h 2 protein required the largest amount of

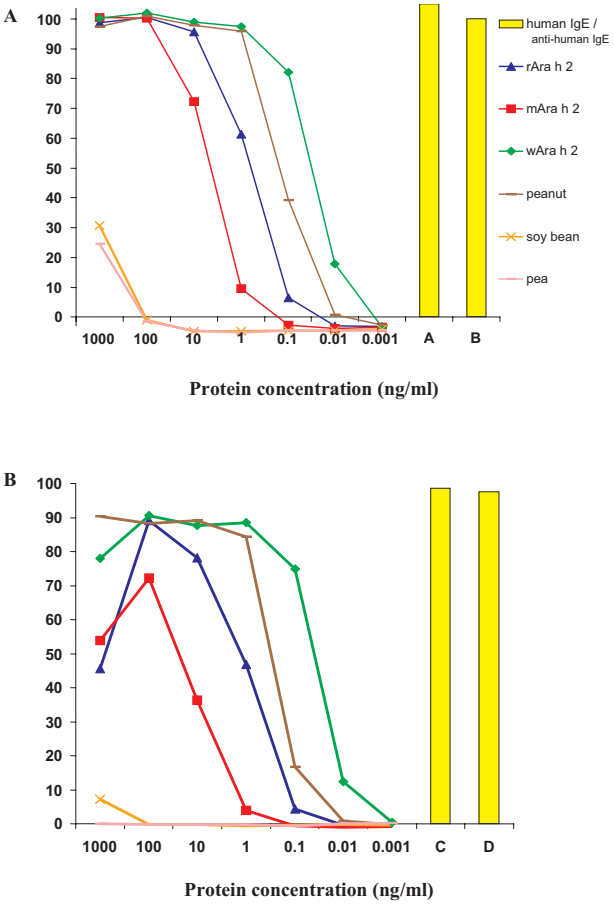

Figure 5. Passively sensitized RBL-2H3 cells release significantly lower amounts of β-hexosaminidase when challenged with the mAra h 2 protein compared to the wAra h 2 protein. RBL-2H3 cells were passively sensitized by incubation with human serum IgE for 18 h at 37°C and 5% CO_2 prior to performing the mediator release assay. Panels A and B represent data collected using sera from two different peanut-sensitive patients. *Y*-axis is expressed as a percent of β-hexosaminidase released. A–D represent positive controls on different plates.

protein (1–10 ng/ml) to elicit 50% β-hexosaminidase release and the Ara h 2 protein isolated from peanuts required the least (0.01 ng/ml).

4 Discussion

Allergen immunotherapy was first attempted at the turn of the century to alleviate patients' symptoms to pollen allergies. Numerous improvements to immunotherapy have been introduced throughout the years, including the method of allergen introduction, better control of allergen purity, and the use of recombinant allergens. Previous studies have shown that $CD4^+$ T-cells from atopic patients produce high levels of IL-4 following stimulation with low concentrations of allergen. Following exposure to high concentrations of allergen, the amount of IL-4 produced by $CD4^+$ T-cells is drastically decreased. The general conclusion from this important finding was that allergens should be administered at high doses if the immune response is going to shift from an IL-4 driven, Th2 allergic response to a Th1 inflammatory response [22].

However, this type of treatment is still not recommended for patients suffering from food allergies due in large part to the severe side-effects and potentially life-threatening anaphylaxis these patients may suffer from the therapy [14]. For recombinant allergens to provide therapeutic value in a clinical setting they need to display a reduced capacity to bind IgE bound to mast cells or basophils, ensuring a lower risk of IgE-mediated side-effects, while retaining their T-cell epitopes, allowing for a modulation of the immune response [23]. Current theory suggests that if a clone encoding an allergenic protein could be mutated to have lower IgE-binding potential, then higher doses of modified protein could be given *per* injection [24].

Many new and innovative treatment options have been suggested for food-allergy immunotherapy including the use of DNA vaccination. Chitosan nanoparticles carrying the gene for Ara h 2 have been used to orally immunize mice against subsequent Ara h 2 sensitization [25]. This treatment was partially successful in inhibiting the development of an allergic sensitization to Ara h 2. Such allergen immunizations could play a role in certain families with a strong genetic predisposition for atopy, however, for the majority of human food allergy, a therapy is needed that is capable of safely and effectively reversing an established atopic immune response. A therapeutic model in which the allergen vaccine is administered to animals that are already sensitized would more closely resemble the human situation.

DNA containing CpG motifs can serve as a potent and relatively safe adjuvant that acts as a strong T helper 1 response inducing substance in mice. However, the type of immune response induced by CpG motifs appears to be mouse strain dependent and will actually heighten the allergic response in some strains [26]. Preliminary phase I and II trials in ragweed allergic patients have shown that allergen–CpG conjugates are well tolerated, less allergenic, and induce IgG antiallergen antibodies more rapidly than allergen extracts alone [27]. The utility of allergen–CpG conjugates for reversing already established allergic responses has yet to be demonstrated.

Our results suggest that it is possible to engineer a hypoallergenic protein that fulfills important criteria for use as an immunotherapeutic agent for allergic disease. A lowered

capacity to bind IgE allows higher doses of allergen to be used with lowered probability of life-threatening side-effects. This was illustrated in our IgE inhibition assay and individual patient IgE binding to the mAra h 2 protein. These assays showed that the single amino acid changes made in each of the Ara h 2 IgE-binding epitopes resulted in significantly lower amounts of IgE binding to this molecule in the majority of patients. However, there was a small cohort of peanut-sensitive patients that showed little or no change in IgE binding to the mAra h 2 protein. This could be due to IgE from these patients recognizing different sequential Ara h 2 epitopes, their IgE recognizing the same epitopes but having different amino acids critical to binding, or their IgE recognizing primarily conformational epitopes. We are currently exploring these possibilities.

Retention of the allergen's ability to interact with T-cells allows for the shift from an allergen-specific Th2 response to an allergen-specific Th1-type response. The observation that the T-cell epitopes from a peanut-sensitive population are polymorphic in nature is not surprising. Other allergens where T-cell epitopes have been mapped in an allergic population show similar results with many regions of the allergen being identified as interacting with T-cells [28–30]. By injecting larger quantities of mAra h 2, there is a better chance that Ara h 2 would be processed by nonprofessional APCs. If processed peptides are presented to circulating T-cells by APCs without the proper costimulatory molecules, then T-cells will become desensitized to that T-cell epitope [31]. Desensitization of the T-cell results in a decrease in IL-4 production, resulting in a decreased capacity to activate B-cells to produce Ara h 2-specific IgE. The mAra h 2 protein retained the ability to stimulate T-cell proliferation from PBMCs obtained from Ara h 2-allergic patients in spite of the fact that our data indicated that many of the T-cell epitopes overlapped with IgE-binding epitopes.

In summary, our results indicate that it is possible to produce a hypoallergenic form of peanut allergen Ara h 2 that binds less allergen-specific IgE, interacts with T-cells from peanut-sensitive patients, and releases significantly lower amounts of mediators from passively sensitized mast cells. Similar attributes have been engineered into peanut allergen Ara h 3 [32]. Since there are three major peanut allergens (Ara h 1, Ara h 2, and Ara h 3) [33], it seems likely that it will be possible to produce hypoallergenic forms of all three allergens, and, when coupled with an appropriate adjuvant, be a potentially safe, effective treatment for peanut allergy.

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5 References

[1] Hanson, L., Telemo, E., *Acta Paediatr.* 1997, *86*, 916–918.

[2] Sampson, H. A., Mendelson, L., Rosen, J. P., *N. Engl. J. Med.* 1992, *327*, 380–384.

[3] Yunginger, J. W., Sweeney, K. G., Sturner, W. Q., Giannandrea, L. A., Teigland, J. D., Bray, M., Benson, P. A. *et al.*, *JAMA* 1988, *260*, 1450–1452.

[4] Burks, A. W., Sampson, H., *Curr. Probl. Pediatr.* 1993, *23*, 230–252.

[5] Sampson, H. A., *J. Allergy Clin. Immunol*. 2004, *113*, 805–819.

[6] Sampson, H., *J. Allergy Clin. Immunol.* 1989, *84*, 1062–1067.

[7] Hourihane, J. O., Roberts, S. A., Warner, J. O., *Br. Med. J.* 1998, *316*, 1271–1275.

[8] Sicherer, S. H., Munoz-Furlong, A., Sampson, H. A., *J. Allergy Clin. Immunol*. 2003, *112(6)*, 1203–1207.

[9] Burks, A. W., Sampson, H. A., *Clin. Rev. Allergy Immunol.* 1999, *17*, 339–360.

[10] Singh, B., Singh, U., *Plant Foods Hum. Nutr.* 1991, *41*, 165–177.

[11] Sicherer, S. H., Sampson, H. A., *J. Allergy Clin. Immunol.* 1999, *104*, 114–122.

[12] Kowalski, M., Jutel, M., *Allergy* 1998, *53*, 485–492.

[13] Durham, S., Till, S., *J. Allergy Clin. Immunol.* 1998, *102*, 157–164.

[14] Oppenheimer, J. J., Nelson, H. S., Bock, S. A., Christensen, F., Leung, D. Y., *J. Allergy Clin. Immunol.* 1992, *90*, 256–262.

[15] Bannon, G. A., Shin, D., Maleki, S., Kopper, R., Burks, A. W., *Int. Arch. Allergy Immunol.* 1999, *118*, 315–316.

[16] Burks, A. W., Williams, L. W., Connaughton, C., Cockrell, G., O'Brien, T. J., Helm, R. M., *J. Allergy Clin. Immunol.* 1992, *90*, 962–969.

[17] Stanley, J. S., King, N., Burks, A. W., Huang, S. K., Sampson, H., Cockrell, G., Helm, R. M. *et al.*, *Arch. Biochem. Biophys.* 1997, *342*, 244–253.

[18] Hoffmann, A., Kaul, S., Fotisch, K., Danz, N., Luttkopf, D., Hatahet, L., Kastner, B. *et al.*, *Arb. Paul Ehrlich Inst.* 2003, *94*, 76–85.

[19] Hoffmann, A., Vieths, S., Haustein, D., *J. Allergy Clin. Immunol.* 1997, *99*, 227–232.

[20] Maeyama, K., Hohman, R. J., Metzger, H., Beaven, M. A., *J. Biol. Chem.* 1986, *261*, 2583–2592.

[21] Schwartz, L. B., Lewis, R. A., Seldin, D., Austen, K. F., *J. Immunol.* 1981, *126*, 1290–1294.

[22] Secrist, H., KeKruyff, R., Umetsu, D., *J. Exp. Med.* 1995, *181*, 1081–1089.

[23] Ferreira, F., Ebner, C., Kramer, B., Casari, G., Briza, P., Kungl, A., Grimm, R. *et al.*, *FASEB J.* 1998, *12*, 231–242.

[24] Marshall, G., Davis, F., *Nat. Biotechnol.* 1997, *15*, 718–719.

[25] Roy, K., Mao, H. Q., Huang, S. K., Leong, K. W., *Nat. Med.* 1999, *5*, 387–391.

[26] Horner, A. A., Takabaysahi, K., Zubeldia, J. M., Raz, E., *Allergy* 2002, *57*, 24–29.

[27] Spiegelberg, H. L., Horner, A. A., Takabayashi, K., Raz, E., *Curr. Opin. Allergy Clin. Immunol.* 2002, *2*, 547–551.

[28] Wurtzen, P. A., Bufe, A., Wissenbach, M., Madsen, H. O., Ipsen, H., Arnved, J., Van Neerven, R. J., *Clin. Exp. Allergy* 1999, *29*, 1614 – 1625.

[29] Texier, C., Herve, M., Pouvelle, S., Menez, A., Maillere, B., *Int. Immunol.* 1999, *11*, 1313 – 1326.

[30] Abe, Y., Kimura, S., Kokubo, T., Mizumoto, K., Uehara, M., Katagiri, M., *J. Clin. Immunol.* 1997, *17*, 485 – 493.

[31] Clark, E., Ledbetter, J., *Nature* 1994, *367*, 425 – 438.

[32] Rabjohn, P., West, C. M., Connaughton, C., Sampson, H. A., Helm, R. M., Burks, A. W., Bannon, G. A., *Int. Arch. Allergy Immunol.* 2002, *128*, 15 – 23.

[33] Burks, W., Helm, R., Stanley, S., Bannon, G. A., *Curr. Opin. Allergy Clin. Immunol.* 2001, *1*, 243 – 248.

