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Redefining the major peanut allergens

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Abstract

Food allergy has become a major public health concern in westernized countries, and allergic reactions to peanuts are particularly common and severe. Allergens are defined as antigens that elicit an IgE response, and most allergenic materials (e.g., pollens, danders, and foods) contain multiple allergenic proteins. This has led to the concept that there are “major” allergens and allergens of less importance. “Major allergens” have been defined as allergens that bind a large amount of IgE from the majority of patients and have biologic activity. However, the ability of an allergen to cross-link complexes of IgE and its high-affinity receptor FcεRI (IgE/FcεRI), which we have termed its allergic effector activity, does not correlate well with assays of IgE binding. To identify the proteins that are the most active allergens in peanuts, we and others have employed in vitro model assays of allergen-mediated cross-linking of IgE/FcεRI complexes and have demonstrated that the most potent allergens are not necessarily those that bind the most IgE. The importance of a specific allergen can be determined by measuring the allergic effector activity of that allergen following purification under non-denaturing conditions and by specifically removing the allergen from a complex allergenic extract either by chromatography or by specific immunodepletion. In our studies of peanut allergens, our laboratory has found that two related allergens, Ara h 2 and Ara h 6, together account for the majority of the effector activity in a crude peanut extract. Furthermore, murine studies demonstrated that Ara h 2 and Ara h 6 are not only the major elicitors of anaphylaxis in this system, but also can effectively desensitize peanut-allergic mice. As a result of these observations, we propose that the definition of a major allergen should be based on the potency of that allergen in assays of allergic effector activity and demonstration that removal of that allergen from an extract results in loss of potency. Using these criteria, Ara h 2 and Ara h 6 are the major peanut allergens.

Keywords

Peanut allergy; Ara h 2; Ara h 6; Recombinant allergen; Basophils

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Introduction

Allergy to peanuts, an IgE-mediated food allergy, has become a major public health concern particularly in westernized countries [1, 2]. The peanut, *Arachis hypogaea*, is a member of the legume family and is related to beans and peas, but not to tree nuts, although up to 50 % of peanut-allergic patients are also sensitized to tree nuts [3, 4]. Immediate hypersensitivity reactions to peanuts are of note in that they are much more prevalent and severe than for other legumes and, for 80 % of patients, persist into adulthood [5]. For peanut-allergic patients, avoidance currently remains the only viable option, although many promising therapeutic interventions, particularly oral immunotherapy, are under investigation [1, 6, 7].

The term “major allergen” was originally used by Lowenstein to designate allergens that cause sensitization in >50 % of patients and was later redefined by Marsh to include only allergens that elicit an IgE response in>90 % of allergic patients [8–10]. Although it does not appear to have been the purpose of those who defined the term “major allergen,” this term now carries the idea that a specific allergen is responsible for a large amount of the allergenic activity of an extract and is therefore important. The data set used currently to substantiate the importance of a “major allergen” is a variant of those suggested by the WHO/IUIS allergen nomenclature subcommittee in 1994 and includes (1) a prominent band seen with sera from most patients on IgE immunoblots of 1-D gels, (2) activity in basophil histamine release (BHR) assays, (3) activity in competitive ELISA assays, and (4) activity in animal models [11]. Although it is acknowledged that the contribution of the allergen to the total potency of the extract should be demonstrated by absorption studies, this has rarely been done for allergens, especially for food allergens [10, 11]. Of note, the allergen nomenclature subcommittee of WHO/IUIS does not grade allergens based on their importance [12].

Eleven potentially important allergens of peanut have been described and, of these, Ara h 1, Ara h 2, and Ara h 3 have been designated as the major peanut allergens [13–18]. This is an important concept because much current research in the fields of diagnostics and therapeutics is based on the idea that specific proteins in peanuts are the primary or major allergens [19–23]. Examples include efforts to develop peanuts devoid of specific proteins, to develop allergens with inactivated IgE-binding epitopes, to develop in vitro diagnostic and in vivo skin-testing reagents, and to develop immunotherapy [24–31]. Definitive identification of the most clinically relevant peanut allergens is critical for our complete understanding of the mechanisms, whereby peanuts are potent allergens and eventually to understanding how the immune system recognizes these allergens. However, early work from our laboratory raised the question as to whether these major allergens were potent enough to explain the allergenic effector activity found in peanuts [32]. The findings summarized in this review have demonstrated that two highly related 2S albumins, Ara h 2 and Ara h 6, are in fact the major peanut allergens. The purpose of this review is to summarize our experience with peanut allergens and to propose new criteria whereby an allergen is considered to be a “major allergen”.

Peanut allergens

Food allergens belong to a select group of protein superfamilies [33–35]. These are (1) the cupin superfamily (7S and 11S seed storage proteins) including viculins and legumins (glycinins); (2) the prolaminin superfamily characterized by 2S albumins, nsLTPs (nonspecific lipid-transfer proteins), α-amylase, and some protease inhibitors; and (3) the plant defense proteins including Prs (pathogenesis-related proteins), proteases, and protease inhibitors [34, 36].

1. Ara h 1 belongs to the vicilin (7S) family of seed storage proteins. It is a glycoprotein and contains 23 independent IgE-binding epitopes [37]. Three-dimensional modeling performed by Burks and co-workers shows that Ara h 1 forms highly stable homotrimers with allergenic sites clustered into two main regions [38]. Ara h 1 forms a stable 150-kD trimer with the IgE-binding epitopes clustered in the regions of monomer–monomer contact [39]. The internal location of these IgE-binding regions likely explains the relative weak activity of native (trimer) Ara h 1 in cross-linking IgE and the strong binding of IgE to denatured monomers (63 kD on immunoblots) [32, 38].

2. Ara h 2 belongs to the conglutin family of seed storage proteins that is related to the 2S albumin family [34]. It contains 10 independent IgE-binding epitopes stretching throughout the linear structure [14]. There are two isoforms, Ara h 2.01 and Ara h 2.02, and two alleles of each [40, 41]. The larger isoform contains 12 extra amino acids, including a duplication of a strong IgE-binding sequence, DPYSPS and binds more IgE [41]. As a result of these two isoforms, Ara h 2 runs as a 17–19-kD doublet on PAGE [40]. The C-terminus of Ara h 2 has some homology with nsLTPs and with a number of other peanut proteins, specifically Ara h 6, Ara h 7, SSP-1, SSP-2, and conglutinin. A conformational model of Ara h 2 and Ara h 6 shows a virtually perfect overlay, indicating highly similar tertiary structures of the two proteins [42].

3. Ara h 3, a peanut glycinin, belongs to the legumin (11S) family of seed storage proteins. Native glycinin is a 360–380-kD protein constructed from 60-kD monomers that are proteolytically cleaved (posttranslational) to yield, after unfolding in 6M urea and reduction of disulfide bonds by DTT, peptides composed of 14, 16, 25, 28, 42, and 45 kD. IgE-binding epitopes are found in the 14-, 42-, and 45-kD fragments [43].

4. Ara h 4 was originally described as a distinct peanut allergen with high homology to Ara h 3. It is no longer thought to be a distinct allergen [34].

5. Ara h 5 is a member of the profilin family of structural proteins that regulate the polymerization of actin [34]. It is a 14-kD protein and is presented at low levels in peanut extracts [43].

6. Ara h 6 is 59 % homologous to Ara h 2 but is 2–4-kD smaller. It is a heat and digestion stable protein with a molecular weight of ~14.5 kD, a protease-stable core, and allergenic potency similar to that of Ara h 2 [15, 42, 44–46]. Lehmann

and colleagues have recently defined the structure of Ara h 6 and demonstrated resistance to proteolytic treatment [42].

7. Ara h 7 also belongs to the conglutin protein family and is 35 % homologous to Ara h 2. Its predicted size is 15.8 kD [15], and its allergenic properties has not been further characterized.

8. Ara h 8 is homologous to Bet v 1, an important allergen in birch pollen and may account for peanut sensitivity in patients who are allergic to birch pollen [18].

9. Ara h 9 belongs to the nonspecific lipid-transfer proteins (nsLTPs) allergen family and seems to play an important part in peanut allergy for patients from the Mediterranean region [47].

10. Ara h 10 (oleosin 1) and Ara h 11 oroleosins have recently been identified, and their allergenic properties are still uncertain [48, 49].

Standard approaches to identify “major” peanut allergens

The standard approach to identify major peanut allergens is primarily based on immunoblotting with sera from patients with allergic reactions to peanuts. IgE binding to specific bands is identified with enzyme or ^{125}I-labeled antibodies specific for human IgE, and a protein is said to be a major allergen if the binding of IgE is prominent and is frequently found for a group of sera from the patients being studied. RAST inhibition assays are also used (see below). Ara h 1, Ara h 2, and Ara h 3 were identified as the major peanut allergens based on data of this nature [34, 50–52]. Subsequent evidence of the importance of Ara h 1, 2, and 3 taken together as the major allergens has been limited to findings assessing IgE binding to linear epitopes in peptide arrays. One laboratory has reported that patients with IgE that bound to the widest array of peptides of these proteins had more severe clinical histories [53, 54].

In vitro immunochemical assays to assess IgE binding to allergens and their drawbacks

It is important to point out that all in vitro assays of binding of IgE to allergens are measuring some aspects of the immunochemical association of IgE and allergen, but none of these assays measures the ability of the allergen to cross-link IgE/FcεRI complexes on the surface of mast cells or basophils leading to cell activation. Although there are some correlations between binding of IgE to allergens in these assays and the functional activation of mast cells and basophils, in general, the relationships are weak [32, 55].

Immunoblots—Historically, IgE immunoblots have been used to identify peanut allergens and to generate inferences regarding the importance of these allergens. However, there are reasons to question the dependability of this approach. For example, there are antibodies that bind proteins on immunoblots, but not in solution, and reciprocally, there are antibodies that bind proteins in solution, but not on immunoblots. Examples can be found in any catalog-listing antibodies for sale where specific antibodies are useful in immunoblots, but not for immunoprecipitation. In a study of rabbit polyclonal antibodies to c-jun N-terminal kinases, we found antibodies that were quite potent for detection of proteins on immunoblots but were able to immunoprecipitate the ligand only if there was sufficient detergent in the

solution, presumably to expose the appropriate epitope [56]. Furthermore, there are examples of epitopes present in the native configuration of a protein that is lost following denaturation or even by separation of a native protein into subunits following exposure to reducing agents. These are called “conformational epitopes” or “discontinuous epitopes” [51, 57]. Although blotted proteins may refold extensively following removal of detergent, this is not necessarily the case [58]. So, even though there may be prominent binding of IgE to a particular epitope on immunoblotting, there may be much less binding in the fluid phase.

ELISA assays—ImmunoCap® and related assays are ELISA-based tests that detect IgE binding to allergens from crude extracts of allergenic materials adsorbed to a solid phase [59]. Historically, ^{125}I-labeled antibodies were used for detection, and these assays were called Radio-Allergo-Sorbent Tests (RAST tests), and this name persists even though radiation is no longer used. These assays are best used to identify allergen-specific IgE and have recently been updated to examine binding of IgE to specific recombinant proteins [60]. Since the allergenic proteins are absorbed onto plastic, they may not be in their native configuration, and these assays may have some of the same drawbacks as immunoblotting. Nonetheless, these assays have had significant success in that the presence of peanut-specific IgE at a level of >15 kU/L or of Ara h 2-specific IgE of >1.19 kU/L are both indicative of true peanut allergy at these values (98 % specificity for both tests). Of note, the Ara h 2-specific IgE test has higher sensitivity than the test for total anti-peanut IgE (60 vs. 26 %) and measurement of binding to other peanut proteins, including Ara h 1, and Ara h 3 did not have added value [61, 62]. Ara h 6 is not a part of this diagnostic assay [61, 62].

ELISA inhibition—ELISA inhibition assays (also called RAST inhibition assays) measure the ability of a soluble allergen to bind IgE and interfere with IgE binding to an immobilized allergen. In these assays, an allergen is attached to a plastic or other solid surface, and the ability of IgE from a patient to bind to the allergen is measured in an ELISA or RIA format. Addition of a *bone fide* allergen inhibits the signal from the patient serum by binding to the patient’s IgE and interfering with the ability of the IgE to bind to the immobilized allergen. This technique has been used to verify that the protein can bind IgE in a fluid phase, to examine allergens for possible cross-reactivity, to standardize extracts, and to demonstrate relative strengths of binding [63].

Molecular Koch’s postulates

The critical question initially addressed in our studies of peanut allergens has also been addressed by Aalberse: “When is a protein considered to be a major allergen?” [58]. Although Koch’s postulates were originally directed at proving that a microorganism causes a specific infectious disease, these postulates have been recast to refer to cells and molecules that may cause a non-infectious disease state [64–67]. In the setting of allergic disease, a molecule thought to be responsible for causing allergic reactions should cause allergic reactions at an appropriate concentration. Also, an allergenic extract should lose activity if this protein is specifically removed, and the activity should be restored with purified allergen.

To fulfill the molecular Koch's postulates for the peanut allergens, one must first isolate the suspect protein or express it using molecular techniques and then challenge a peanut-allergic person, animal, or a mast cell/basophil system sensitized with allergen-specific IgE with serial dilutions of original CPE and the purified proteins. An in vitro model system such as the RBL SX-38 cell assay or an ex vivo model such as basophil histamine release (BHR) is a reasonable first approximation to an in vivo study. Skin-test titrations (humans) and a murine model of peanut allergy are reasonable in vivo systems. Double-blind, placebo-controlled food challenge (DBPCFC) in peanut-allergic patients is the gold standard, but this is far too cumbersome for studies such as this. Then, one must compare quantitatively the functional activity of the individual allergens with the functional activity in the original extracts and see whether the activity in the original extract can be accounted for by the purified reagent. These proteins, if they are indeed the most clinically important allergens, should account for a significant amount of the activity presents in the extract. In these experiments, it is necessary to demonstrate that the material is pure.

A complementary approach is to remove the putative "major" allergen by immunodepletion, chromatography, or by genetic means and demonstrate that the "cleared" extract has had its allergenicity reduced considerably. Here, it is incumbent on the investigator to demonstrate that the removal of the allergen is complete and specific. An alternative approach is to selectively remove the allergen-specific IgE with a solid-phase allergen and demonstrate complete and specific removal of the allergen-specific IgE. This cannot be done in vivo, but rather must be done in vitro with a cell-based assay such as the RBL SX-38 cell assay or with stripped basophils.

Few investigators have employed these approaches to the study of specific allergens. De Groot et al. [68] depleted an extract of cat dander of Fel d I (by 95 %) with mono-clonal and with polyclonal antibodies. In BHR tests, the depleted extracts were 30–300 times less potent than the original extracts, demonstrating that Fel d I is a major allergen of cat dander [68]. Lombardero et al. depleted an extract of olive pollen of the allergen Ole e I using monoclonal antibodies against two nonoverlapping epitopes. The removal of Ole e I resulted in a large reduction in the allergenic activity as measured by skin tests and BHR [69]. A limitation of these experiments is that the potency of purified allergens was not compared to the predicted contribution to the potency of the crude extracts, the specificity of the immunoprecipitation step was not demonstrated, and add-back experiments were not performed [70]. Of note, similar experiments were performed to remove Der p 1 from a dust mite extract (whole body extract), and there was no effect on the potency of the extract [71]. Norman and colleagues purified Amb a 1 (at that time called antigen e) from ragweed and demonstrated that this allergen alone could effectively desensitize patients with hay fever [72]. This is a strong indication that Amb a 1 is the major allergen of ragweed. Witteman and colleagues found similar potency among purified Der p 1, Der p 2, Fel d 1, Lol p 1, and Lol p 5 (putative major allergens) but did not demonstrate that the potency of any of these allergens was sufficient to account for the activity in the parent extracts [73]. We cannot find evidence that either quantitative determinations of potency relative to the amount of allergen in the parent extract or depletion experiments have ever been performed for any allergens other than Amb a 1, Fel d 1, and Ole e I. Even though it is clear that Ara h 1, Ara h 2, and Ara h 3 elicit a strong IgE response and can cross-link IgE to activate mast cells and

basophils, it has not been proven that Ara h 1 or Ara h 3 can account for a substantial part of the allergic effector function of peanuts.

Redefining the major peanut allergens based on effector function and not on IgE binding alone

We have chosen to use the words “allergic effector activity” instead of “allergenic activity” or “immunoreactivity” because the word “allergenic” and “immunoreactive” are not precise terms. The term “allergenic” can be used to describe the ability of an antigen to elicit an IgE response, to cross-link IgE leading to cell activation, or it may refer to both activities. The term “immunoreactivity” can refer to IgE-allergen binding alone or can be used more generally. We have focused on the allergic effector function of allergens as defined as the ability of allergens to effectively cross-link IgE bound to the high-affinity receptor for IgE, FcεRI, leading to activation of mast cells and basophils. We have further redefined this question of what a major allergen is by specifically asking that peanut allergens account for the majority of the allergic effector function of peanuts.

In vitro and ex vivo, cell-based, functional assays to measure effector function of allergens

RBL SX-38 cells assay—RBL SX-38 cells are rat basophilic leukemia cells that stably express approximately 70,000 copies per cell of α, β, and γ chains of the human high-affinity receptor for IgE, FcεRI [74]. The human receptor gives these cells the important property that they can bind IgE from the sera of allergic individuals and can be activated in an allergen-specific manner [74, 75]. We have used these cells to successfully quantify the ability of the peanut allergens, Ara h 1, Ara h 2, and Ara h 6, to degranulate RBL SX-38 cells following sensitization of the cells with IgE from either a pool of subjects or from individual subjects [32, 76–78]. Other analogs of the RBL SX-38 cells exist and are similarly useful [79].

Basophil histamine release (BHR) assay—Basophils from allergic individuals are the most readily available analog of sensitized human mast cells. Since basophils are the only circulating cells that bind significant amounts of IgE and are the only circulating cells that contain histamine, the physiological effects of allergen/IgE interactions can be assessed in unpurified white blood cells preparations. Significant drawbacks to using basophils are that sensitized basophils are easily activated, must be studied soon after phlebotomy, and are non-functional in 8–20 % of subjects [80, 81]. An alternative to measuring histamine release is to monitor expression of CD63 or CD203c on the basophil surface by flow cytometry [82]. Basophils from allergic donors who are not peanut allergic can be stripped of their IgE and sensitized in the same way we are sensitizing the RBL SX-38 cells [83]. However, stripping IgE is cumbersome. An alternative is to sensitize basophils from a non-allergic donor with serum from a peanut-allergic patient. The use of basophils requires frequent phlebotomy of the donor and does not generate cells with the relative reproducibility of the RBL SX-38 cells.

In vivo (functional) assays to measure effector activity of allergens

Oral challenges—Oral challenges to assess activity of peanut allergens in humans are not feasible because of ethical concerns. The commonly used murine model is the peanut-allergic female C3H/HeJ mouse that is sensitized with freshly ground whole peanut with cholera toxin as an adjuvant [84]. These mice develop IgE antibodies and T-cell proliferative responses to Ara h 1 and Ara h 2 similar to those in peanut-sensitive humans [84]. The IgE antibodies produced in these mice to Ara h 2 recognize 9 of 11 linear epitopes that are recognized by naturally occurring human anti-Ara h 2 IgE [84].

Skin tests—Skin testing has the advantages that it is much less risky than oral allergen challenge and can theoretically be performed in humans, the test subject of choice. Furthermore, multiple doses can be tested on a given individual. This has not been done in the United States because the FDA requires an Investigational New Drug (IND) certificate for any purified materials to be used for skin tests in humans (personal communication, FDA). However, such an approach has been allowed in Europe [55]. Of note, Koppelman found a reasonable correlation between BHR and skin test titration [43]. Skin-test titrations could be performed in the animal model, but this has not been commonly reported.

Determining the major peanut allergens

Effector activity of purified allergens

We and others have demonstrated that Ara h 2 and/or Ara h 6 is more potent than Ara h 1 and/or Ara h 3 using IgE from patients to sensitize RBL SX-38 cells and/or with skin prick tests in peanut-allergic subjects [32, 55, 85, 86]. Of these allergens, only Ara h 2 and Ara h 6 appear to be highly potent in assays of allergic effector activity [32, 42, 46, 55, 76, 78, 85, 87]. In fact, a recent study of peanut varieties with markedly reduced Ara h 1 content found no diminution of the ability of these peanuts to activate RBL cells sensitized with peanut-specific IgE [88].

Effector activity of 20-kD fraction

In an early study, crude peanut extracts were fractioned by gel-filtration chromatography, and effector activity was assayed by measuring degranulation of RBL SX-38 cells sensitized with IgE from individual sera and from pools of sera of peanut-allergic subjects. We found that the majority (80–90 %) of the effector activity of a crude peanut extract (CPE) from gel filtration resides in a single fraction comprised of components in the 13–25-kD range, which we refer to as the 20-kD fraction [76]. When all individual fractions excluding the 20-kD fraction were recombined, the remained effector activity was only ~10 % of the original extract. Two-dimensional (2D) gel electrophoresis of this 20-kD fraction revealed more than 60 protein spots, and, unexpectedly, mass spectrometry revealed that>97 % of this biologically active fraction consisted of Ara h 2, Ara h 6, and variants of these proteins [76]. This suggested that Ara h 2 and Ara h 6 account for the majority of the effector activity found in a crude peanut extract. In further studies, we have found that Ara h 2 and Ara h 6 are substantially redundant in their contribution to the allergic effector activity of CPE [46].

Removal of Ara h 2 and Ara h 6 independently from a CPE

Since Ara h 2 is more potent than Ara h 1 and Ara h 3 and the 20-kD fraction containing Ara h 2 and Ara h 6 accounts for 80–90 % of the allergic effector activity [32, 76], we reasoned that Ara h 2 was the best candidate for the peanut allergen that may account for a substantial amount of the allergic effector function of peanuts, in the same way that Fel d 1 appears to be responsible for most of the allergic effector function of an extract of cat dander. We hypothesized that if Ara h 2 is a major allergen, we should find diminution of activity in an extract that has been fully depleted of Ara h 2. For these experiments, we raised anti-peptide anti-Ara h 2 antibodies and found that one of the rabbit anti-peptide antibodies (against the C-terminal peptide of Ara h 2, CDLEVESGGRDRY) was able to bind to Ara h 2 in solution and specifically and quantitatively (>99 %) remove it from the CPE [78]. Unexpectedly, the Ara h 2-depleted CPE was measurably, but not dramatically less potent than the sham-treated control CPE in its ability to cross-link IgE and activate the RBL SX-38 cells sensitized with IgE from either a pool of subjects or from individual subjects [78]. Clearly, the removal of Ara h 2 does not have the same effect of removal of Fel d 1. Our best estimate of the actual contribution of Ara h 2 to the total effector activity in the CPE ranged from 0 to 40 % of the total activity depending upon the individual sera (from highly peanut-allergic subjects) with a mean value of 21 % [78].

Removal of both Ara h 2 and Ara h 6 from a CPE

Since Ara h 2 and Ara h 6 together account for the majority of the allergic effector activity found in a crude peanut extract and the removal of Ara h 2 from a crude peanut extract (CPE) minimally altered the allergic effector activity of the extract, we hypothesized that Ara h 6 might contribute significantly to this activity. We generated specific rabbit anti-peptide antibodies and found that one of the rabbit anti-peptide antibodies (against the unique peptide of Ara h 6, EQEQYDSYDIRSTRSSDQ) was consistently capable of removing Ara h 6 without removing Ara h 2 [77]. Using this antibody and the antibody previously described for Ara h 2, we removed Ara h 2 and Ara h 6 selectively or simultaneously from CPE by immunodepletion. Immunoblot analysis confirmed that each protein was specially depleted by ~99 % when assessed either with rabbit-peptide antibodies or with human anti-peanut IgE [77]. RBL SX-38 cells sensitized with IgE either from pooled sera or individual sera from peanut-allergic donors were then stimulated with various doses of either untreated CPE, control CPE, or CPE depleted of Ara h 2, Ara h 6, or both Ara h 2 and Ara h 6. We found that removal of Ara h 2 or Ara h 6 alone did not significantly reduce the effector activity of peanut extract. However, immunodepletion of both Ara h 2 and Ara h 6 together significantly reduced the effector activity of CPE [77]. These data are consistent with what was seen when the 20-kD fraction (containing both Ara h 2 and Ara h 6) was removed from the CPE by gel filtration and suggested that these proteins together are the major peanut allergens.

Ara h 2 and Ara h 6 are the major elicitors of anaphylaxis and can effectively desensitize peanut-allergic mice

We further asked if Ara h 2 and Ara h 6 together are the primary peanut allergens responsible for allergic reactions in vivo. To do this, we used an oral sensitization C3H/HeJ

mouse model of peanut allergy to assess the activity of Ara h 2 plus Ara h 6 (Ara h 2/6) (as found in the 20-kD fraction) and CPE without the 20-kD fraction (CPE w/o 20 kD (no Ara h 2 or Ara h 6)) for allergen challenge and immunotherapy [87]. The activity of these preparations was also determined in basophil histamine release assay in whole blood from peanut-allergic subjects. Compared with mice challenged with control CPE, mice challenged with CPE w/o 20 kD experienced reduced symptoms and a smaller decrease in body temperature [87]. Basophil histamine release assay also shown that CPE depleted of Ara h 2 and Ara h 6 dramatically reduced the ability to release histamine in whole blood from peanut-allergic donors, which corroborated the above findings in the mouse model [87].

Since the 20-kD fraction appeared to play an important role in inducing anaphylaxis, it was important to study whether this preparation could desensitize peanut-allergic mice and prevent allergic reactions to peanut. The peanut-allergic mouse model was administered with CPE, Ara h 2/6 (20 kD), CPE w/o 20 kD, or placebo in an immunotherapy protocol. Compared with placebo treatment, peanut-allergic mice treated with 20-kD fraction experienced significantly reduced symptoms, changes in body temperature, and release of mast cell protease 1 (MMCP-1) [87]. In addition, treatment with the 20-kD fraction (Ara h 2/6) was as effective as immunotherapy with complete CPE, whereas CPE w/o 20 kD was significantly less effective for higher-dose peanut challenges [87]. Thus, we conclude that Ara h 2 and Ara h 6 together are the most potent peanut allergens in vivo and when used in immunotherapy are sufficient to prevent allergic reactions to a complete CPE.

Summary of findings regarding the major peanut allergens

As summarized in Table 1, we have shown that, for Ara h 1 and Ara h 2, assays of FcεRI/IgE cross-linking (e.g., RBL SX-38 and BHR) do not correlate with assays of IgE binding (e.g., immunoblots and ImmunoCaps®) [32]. To identify the proteins that are the most active allergens in peanut extracts, we have demonstrated that clinically important allergens should be identified by their ability to functionally cross-link IgE and not just by their ability to bind IgE.

It appears that Ara h 2 is an “important” peanut allergen in that it accounts for 0–40 % of the effector activity of a CPE depending upon patients, but it does not account for the majority of the effector activity in a CPE for any patient examined to date [78]. To address the question of which peanut allergens account for the majority of the allergic effector activity, crude peanut extracts were fractioned by gel-filtration chromatography, and effector activity was measured with the RBL SX-38 cell assay. We found that the Ara h 2 and Ara h 6, co-purified together in a 13–25-kD fraction (20-kD fraction) on gel filtration, account for the majority of effector activity [76]. To address the contribution of individual allergens to a complex crude peanut extract, we developed specific rabbit anti-peptide antibodies to remove peanut allergens, Ara h 2 or Ara h 6. Although Ara h 2 and Ara h 6 can be exclusively removed from a CPE, removal of each alone from a CPE had a minimal effect on the effector function, which suggested that there may be no single major peanut allergen [46, 78]. However, removal of Ara h 2 and Ara h 6 together diminished the major effector activity of CPE [76]. These findings were corroborated when the 20-kD fraction (Ara h 2/6) was removed from CPE by gel filtration and then repleted with either purified Ara h 2 or

Ara h 6 [46]. This further confirmed that both of these proteins are important peanut allergens and that neither independently meets our definition of a major allergen, although together they do meet the definition.

Further studies on murine model of peanut allergy demonstrated that Ara h 2 and Ara h 6 are the major elicitors of anaphylaxis [87]. The basophil histamine release assay (BHR) using whole blood from peanut-allergic subjects also demonstrated a significant effect of removal of Ara h 2/6. Both murine and BHR data confirmed the findings using RBL SX-38 cells that the majority of the effector function of CPE is attributable to Ara h 2 and Ara h 6 (20-kD fraction). Futhermore, desensitization with 20-kD fraction produced an equivalent effect as desensitization with the parent CPE, and either the 20-kD fraction or CPE were significantly better than CPE w/o 20 kD [87].

Proposed criteria to determine the “major” allergens

Taken together, by identifying the ability of allergens to functionally cross-link IgE and not just by the ability of allergens to bind IgE, we propose that the definition of a “major” allergen be based on findings that the activity of the allergen be sufficient to explain>50 % of the activity in the parent extract and that this be true with IgE from >50 % of affected patients. This should be substantiated by depletion and repletion experiments (Table 2). This definition of “major” allergens has important diagnostic and therapeutic implications.

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Abbreviations

20-kD fraction	Gel-filtration chromatography fraction containing Ara h 2 and Ara h 6
Ara h 2/6	Ara h 2 plus Ara h 6 as found in the 20-kD fraction
BHR	Basophil histamine release
CPE	Crude peanut extract
MMCP-1	Mouse mast cell protease 1

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Table 1

Summary of experiments that demonstrate that Ara h 2 and Ara h 6 together are the major peanut allergens

Experimental approach	Findings
Assess potency of purified Ara h 2 and Ara h 6	Ara h 2 is ~4 % of the peanut proteome and has 10 times the specific activity of CPE [46] Ara h 6 is ~6 % of the peanut proteome and has 8 times the specific activity of CPE [46]
Remove Ara h 2 by immunoprecipitation	CPE depleted (> 99 %) of Ara h 2 is only slightly less potent than the original CPE [89]
Remove Ara h 6 by immunoprecipitation	CPE depleted of Ara h 6 has similar potentcy as the original CPE [77]
Remove both Ara h 2 and Ara h 6 by immunoprecipitation	CPE depleted of both Ara h 2 and Ara h 6 has significantly reduced activity (~20 %) compared with the original CPE [77]
Remove Ara h 2 and Ara h 6 together by gel-filtration chromatography	CPE depleted of both Ara h 2 and Ara h 6 (CPE w/o 20-kD fraction) has significantly less activity (~10–20 %) compared with the original CPE [76]
Replete CPE w/o 20-kD fraction with purified Ara h 2 and Ara h 6	Either Ara h 2 or Ara h 6 can replete 80 % of the activity lost by removing both Ara h 2 and Ara h 6 [46]
Assess contribution of Ara h 2 and Ara h 6 together in eliciting anaphylaxis in peanut allergy murine model	Ara h 2 and Ara h 6 are the major elicitors of anaphylaxis [87]
Assess potency of Ara h 2 and Ara h 6 together to desensitize peanut-allergic mice	Desensitization with Ara h 2 and Ara h 6 together produced an equivalent effect as desensitization with the parent CPE [87]. This experiment has not been performed with either Ara h 2 or Ara h 6 alone

Table 2

Proposed new criteria for designating an allergen(s) as a major allergen(s)

Item	Criteria for designating an allergen(s) as a major allergen(s)
Potency	Activity of purified allergens in a biologic assay of cross-linking IgE/FceRI complexes is sufficient to explain > 50 % of the activity in the whole extract for > 50 % of allergic subjects
Depletion	Allergen depletion leads to a significantly reduction (> 50 %) of the total effector activity
Repletion	Purified allergen can replete the activity of an extract that has been selectively depleted of the allergen to be tested
Model system	Key experiments should be performed both in vitro (BHR or similar models) and in vivo (animal models or skin tests in humans)