

Characterization of allergenic food proteins for improved diagnostic methods

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Purpose of review

Diagnostic decision points for food allergen-specific serum IgE concentration and for skin prick test results have been established for several foods, reducing the requirement for food challenges in a number of patients. Many patients, however, still need to undergo oral food challenges because their food-specific IgE level is in the undefined range. In addition, diagnostic decision points could not be established for several foods. It appears that measurement of serum specific IgE concentrations to individual allergens is superior to determination of specific IgE to the crude food extract containing allergenic and nonallergenic proteins. This review will outline recent advantages in characterization of food allergens as well as the relevance of this knowledge for use in recently developed protein microarray technology.

Recent findings

Protein microarrays have been developed to profile allergen-specific IgE antibodies from human serum with the advantage of screening hundreds of allergens in parallel using minute amounts of blood. This technology, however, requires prior knowledge of the proteins to be studied. The identification and characterization of clinically relevant allergens have increased dramatically within the last several years. Relevant new allergens have been identified, especially in tree nuts and seeds. Interestingly, most of these allergens belong to the same family of seed storage proteins. In addition, known food allergens have been further characterized and IgE-binding sites have been determined. Moreover, 'informative' peptides shown to be predictive for the persistence of food allergy have been identified.

Summary

The combination of food allergen characterization and protein or peptide microarray technology will enable us to develop improved diagnostic tools in food allergy.

Keywords

food allergy, allergens, diagnostic, IgE, Proteomics, microarray

Abbreviations

DBPCFC double blind, placebo-controlled food challenge
LTP lipid transfer protein

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Introduction

Allergic reactions to food affect up to 8% of children in the first years of life and 2% of the population overall, with milk, egg, peanut and tree nuts, fish and shellfish accounting for most of the documented allergic responses. Other foods such as sesame seeds, however, appear to be an increasing problem [1–3,4•]. The majority of food allergic reactions are IgE-mediated [5] and the diagnosis of food allergy is normally based on history, skin prick test or in-vitro measurement of specific IgE in the serum, exclusion diets and double blind, placebo-controlled food challenges (DBPCFCs) [6]. DBPCFC remains the gold standard in food allergy diagnosis, however the procedure is time consuming, costly and places a great deal of stress on the patient. In addition, it places the patient at risk for a severe anaphylactic reaction. Therefore, the search for in-vitro diagnostic tests of food allergy is ongoing. In-vitro tests need to be developed for predicting the patient's current state of reactivity and to assess the likelihood of eventual tolerance. The latter is especially important to select children for whom immunotherapy would be of benefit in the future.

Recently, food allergen-specific IgE concentrations in the serum and skin prick test wheal diameters have been correlated with patient histories and the outcome of DBPCFC in order to generate probability curves that depict the likelihood of patients with a particular food allergen-specific IgE level reacting to the food. Diagnostic decision points for food allergen-specific serum IgE concentration and for skin prick test results have been described for peanut, egg, milk and fish [7,8,9•,10,11,12•,13]. These diagnostic decision points help to reduce the requirement for food challenges in a number of patients. However, several problems are still not solved: (1) The diagnostic decision points varied widely between the different study populations. This might be explained by differences in age and atopy status between the various study populations and can be solved through additional studies defining age and disease-related diagnostic decision points. (2) Many patients still

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require an oral food challenge because their IgE levels or skin prick test wheal diameters are in the undefined range, and, with decreasing IgE levels, patients need to undergo oral food challenge in order to determine if they developed tolerance. (3) For several foods, for example wheat and soy, diagnostic decision points could not be established at all. This suggests that the crude allergen extracts used in the tests are not optimal for allergy diagnosis. The fact that IgE antibody to wheat allergen ω -5 gliadin was recently shown to correlate very well with oral challenge results [14] points in this direction. Similar results were found in Brazil nut allergy, where sensitization to Ber e 1 was shown to correlate with the clinical expression of the allergy [15]. The use of purified or recombinant allergens, instead of whole food allergen extracts commonly used for skin prick tests or measurement of specific serum IgE, might give more accurate results in food allergy diagnostic methods.

Improved diagnostic methods

Determination of allergic sensitization is normally done by skin prick test *in vivo* or determination of allergen-specific serum IgE *in vitro*. The recent emergence of protein microarray technology may provide significant advances [16•]. This technology involves the application of hundreds of different proteins, in tiny quantities each, to the surface of treated slides so that essentially hundreds of assays can be run in miniature.

Comparison of protein microarray technology with standard techniques

The first comparisons between microarray-based immunoassays and the Pharmacia CAP System (Pharmacia Diagnostics, Kalamazoo, MI, USA) showed a good correlation for food and aeroallergens [16•,17•]. Microarray assays have at least three important advantages over currently used in-vitro determination of IgE. First, they can screen hundreds of allergens in parallel and second, they only require minute amounts of serum. Especially in pediatric patients, the requirement of less than 50 μ l of sera for simultaneous detection of hundreds of allergens will revolutionize clinical diagnostic and research efforts. Moreover, microarray assays require much less allergen than in-vitro tests currently used. This is important for the use of individual purified or recombinant allergens that are usually more expensive and difficult to obtain.

Use of purified or recombinant proteins in protein arrays

Another step forward was demonstrated by the use of individual allergen microarrays as a novel method for the diagnosis of type I allergy [18•]. Recombinant or purified allergen molecules were spotted on glass slides to obtain allergen chips that allow the determination of patients' reactivity profiles with minute amounts of

serum in single tests [18•]. One major difference between the allergen microarray described in this study and currently used tests is that the microarray contains defined allergens as reactants, whereas currently used tests are based on allergen extracts containing a mixture of allergenic and nonallergenic molecules.

Use of peptide arrays in allergy diagnosis

The particular segments of food protein that are recognized by IgE antibody (epitopes) have been identified for several food allergens, but their diagnostic importance is not well understood. Currently, epitope analysis is carried out with protein digests or membrane-based immunoassays. These methods are time consuming and require large quantities of patient sera. Therefore, we recently developed a peptide microarray for the analysis of IgE-binding epitopes [19].

Characterization of allergenic food proteins

Although microarray technology may improve the in-vitro diagnosis of food allergy, it requires information about the relevant allergens or allergenic peptides (IgE binding sites or epitopes). For some foods, such as milk, egg, fish, shellfish and peanut, the major allergens have been identified. In addition, through recent advances in Proteomics and molecular biology, identification of new allergens for several foods such as tree nuts and seeds could be performed. Moreover, the structure of many food allergens as well as their IgE-binding sites has been characterized within the last several years. In contrast to aeroallergens in which IgE-binding occurs primarily to conformational epitopes located on the surface of the protein, it appears that for food allergens sequential (linear) epitopes also play an important role. This might be explained by the fact that sequential epitopes are more likely to survive digestion intact (Fig. 1).

Cow's milk

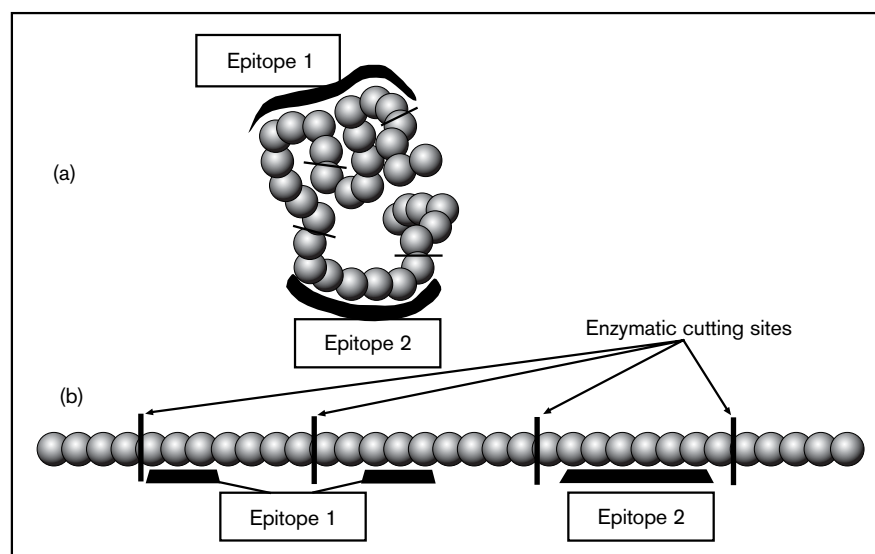
Caseins appear to be the major allergenic milk proteins in man and account for 80% of the total protein in cow's milk [20]. They are composed of four fractions with little primary structure homology: α_{s1} , α_{s2} , β , and κ -casein constituting 32%, 10%, 28%, and 10% of the total protein content, respectively [21]. Whey proteins constitute 20% of total milk proteins and comprise the allergens α -lactalbumin and β -lactoglobulin. We have recently identified the IgE-binding epitopes for all casein and whey allergens [22–25]. Moreover, we have shown that specific 'informative' epitopes were predictive for the natural course of cow's milk allergy [26•].

Hen's egg

Ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferin (Gal d 3), and lysozyme (Gal d 4) have been identified as major hen's egg allergens [27]. Ovomucoid appears to be the dominant allergen in hen's egg, and children with

Figure 1. A hypothetical protein in (a) folded and (b) linearized form

Epitope 1 represents a conformational and epitope 2 a sequential (linear) IgE-binding site. Potential enzymatic cutting sites highlight the fate of these epitopes after digestion.



persistent egg allergy have significantly higher concentrations of IgE anti-ovomucoid antibodies than those who 'outgrow' their reactivity [28]. The allergenic (IgE-binding) and antigenic (IgG-binding) B cell epitopes of ovomucoid have been identified [29–31]. In addition, in contrast to Gal d 2, Gal d 3 and Gal d 4, ovomucoid is relatively resistant to treatment by heat or digestive enzymes. It had been suggested that measurement of ovomucoid specific IgE antibodies might be useful in distinguishing between patients who can tolerate heat-treated eggs from those who cannot [32]. Moreover, IgE-binding activity to pepsin-digested ovomucoid was of diagnostic value in distinguishing challenge-positive from challenge-negative individuals [33]. Analyzing various prognostic factors for the development of tolerance in hen's egg allergy, it was shown that specific IgE antibody levels are important prognostic markers in children with cutaneous symptoms [34•]. As seen in milk allergy [26•], we recently found an association between a child's allergenic epitope recognition on ovomucoid and the natural history of their hen's egg allergy (K.M. Jarvinen *et al.*, unpublished data).

Peanut

For peanut allergy, the major and minor allergens have been identified; they comprise the peanut congenitins Ara h 2, 6 and 7 [35,36], the peanut vicilin Ara h 1 [37], the peanut glycinins Ara h 3/4 [36,38] and the peanut profilin Ara h 5 [36] (Table 1). The IgE binding sites for Ara h 1, 2 and 3 have been determined using SPOTS peptide synthesis technology [35,38–40]. We recently compared a peptide microarray for the analysis of IgE-binding epitopes of the three major peanut allergens, Ara h 1,

Ara h 2 and Ara h 3, with the well established SPOTS membrane method using 210 peptides, 20 amino acids in length [19]. We found a high degree of correlation between peptides recognized using this method with the previously identified epitopes. In parallel, we determined the binding of serum IgE to recombinant Ara h 1, Ara h 2 and Ara h 3 in 90 patients with peanut allergy using the microarray technique as shown in Fig. 2. Forty-nine percent of the patients recognized all three allergens, 8% Ara h 1 and 2, 11% Ara h 1 and 3, 10% Ara h 2 and 3, whereas 7%, 4% and 4% recognized only Ara h 1 or Ara h 2 or Ara h 3, respectively (Fig. 2) (WG Shreffler *et al.*, unpublished data). This distribution of allergen recognition is similar to that previously published using methods other than protein microarray [36].

Very recently it has been shown for Ara h 2 that the protein structure plays a critical role in the stability of this peanut allergen. Enzymatic digestion results in a 10 kDa protease-resistant fragment that contains multiple intact IgE-binding epitopes [41•]. It can be speculated that IgE recognition of this protease-resistant protein fragment or of the IgE-binding peptides located on this fragment, combined with similar measurements for the other peanut allergens, have better correlation to challenge outcome than determination of specific IgE to crude peanut extract.

Soybean

Several soybean allergens have been described, with the major allergens appearing to be various seed storage proteins including glycinin G1 and G2, and β -conglycinin (Table 1) as well as the soy profilin Gly m 3 [27].

Table 1. 2S-Albumin, 7S and 11S-globulin seed storage proteins

	2S Albumins Prolamin superfamily	7S Globulins (vicilins) Cupin superfamily	11S Globulins (legumins)
Peanut		Ara h 1 [37]	Ara h 3/4 [36,38]
Soybean		β -Conglycinin	Glycinin
English walnut	Jug r 1 [46]	Jug r 2 [45]	
Black walnut	Jug n 1 [47]	Jug n 2 [47]	
Hazelnut	+ ^a [49•]	Cor a 11 ^b [52]	Cor a 9 [48•]
Brazil nut	Ber e 1 [15]		+ ^c
Cashew nut	+ [54]	Ana o 1 [55•]	Ana o 2 ^d [54]
Almond	+ ^a [56]	+ ^a [56]	
Sesame seed	Ses i 1 [66] + Ses i 2 [61••]	Ses i 3 [61••]	
Mustard seed	Sin a 1 [62] + Bra j 1 [63]		

^aFor these allergens short N-terminal amino acid sequences were obtained that showed homology to 2S albumins or 7S globulins, respectively.

^bAF441864.

^cK. Beyer *et al.*, unpublished data.

^dAAN76862.

IgE-binding sites for several of these allergens have been identified and it has been shown that the soybean glycinin G1 acidic chain shares IgE-binding epitopes with the peanut allergen Ara h 3 [42]. In addition to these allergens, a Bet v 1-related PR-10 protein, SAM 22, has been identified recently in birch pollen-allergic patients with immediate reactions to soy [43].

Soy-based formulas are sometimes used as cow's milk substitutes in the treatment of cow's milk allergy. Recently, a 30 kDa component from soybean that cross-reacts with cow's milk caseins has been identified as the A₅B₃ glycinin molecule [44].

Tree nuts and almond

The characterization of tree nut allergens began much more recently. Major and minor allergens have been identified in English walnuts [45,46], black walnuts [47], hazelnuts [48•,49•,50–52], Brazil nuts [15,53], cashew nuts [54,55•] and almonds [56]. In addition, a model of the three-dimensional structure of the Brazil nut allergen Ber e 1, a 2S albumin, was recently generated [57].

Interestingly, most of these allergens belong to few protein families. The 'tree-pollen-unrelated allergens', responsible for systemic reactions, belong to the family of seed storage proteins, as seen with the peanut allergens (Table 1). Other allergens belong to the pathogenesis-related proteins including the lipid transfer proteins (LTPs) in hazelnut, Cor a 8 [51], and walnut, Jug r 3. LTPs were identified as allergens in fruits of the Rosaceae family; however, recent studies demonstrated immunological cross-reactivity between LTPs from different botanical families [58,59].

To date, IgE-binding sites have been determined for the walnut allergen Jug r 1, a 2S albumin, and the cashew nut allergen Ana o 1, a 7S globulin. For Jug r 1,

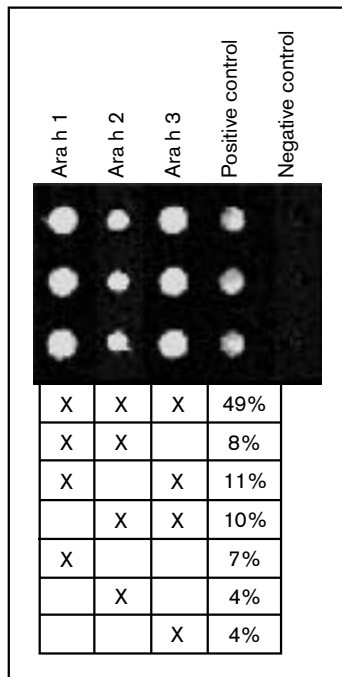
one major linear IgE-reactive epitope and its critical core amino acids have been identified [60•]. For Ana o 1, epitope mapping revealed 11 linear IgE-binding epitopes, of which three appeared to be immunodominant [55•].

Although botanically unrelated, proteins within the same group of seed storage proteins show a high degree of homology. As an example, Fig. 3 shows the alignment of several 7S globulins for two regions, one representing an area with a high degree of homology and the second with a low degree. In addition, the IgE-binding regions in these areas for Ara h 1 and Ana o 1 are indicated. Cross-allergenicity between proteins might occur if IgE-binding regions show a high degree of homology. In general, the cashew allergen Ana o 1 and the peanut allergen Ara h 1 have 45% similarity and four epitope regions showed positional overlap. Among the overlapping epitopes, however, no significant homology is found between Ana o 1 and Ara h 1 sequences [55•]. In contrast, one of the known IgE-binding epitopes of Ara h 1 has a high degree of homology with the corresponding area of the sesame vicilin, Ses i 3 [61••] (Fig. 3). The amino acids that differed were previously shown not to be critical for IgE binding to Ara h 1 using single amino acid substitution. Similarly, we have shown that IgE-binding epitopes of the peanut allergen Ara h 3 has 67% identity with the corresponding areas of the hazelnut allergen Cor a 9, both 11S globulins [48•]. Again, the amino acids that differed were previously shown not to be critical for IgE-binding to this peanut protein. This would suggest a high likelihood that patients would possess cross-reactive antibodies, which could provoke clinical symptoms.

Seeds

Similar as in tree nut allergy, more and more allergens are detected in various seeds. The major allergens for

Figure 2. Recombinant Ara h 1, Ara h 2 and Ara h 3, positive and negative controls were spotted in triplicate onto poly-L-lysine slides, 375 μ m apart



For detection of IgE binding, immunolabeling was performed with individual patient sera from 90 peanut allergic individuals. Nonpeanut allergic human serum served as a negative control. After incubation with biotinylated anti-human IgE antibody and Avidin-Cy3, fluorescence was measured using a GeneArray Reader. Shown in this figure is the labeling of one of the peanut allergic patients. The table underneath the figure gives the percentage of individuals recognizing the indicated allergen combination (W.G. Shreffler *et al.*, unpublished data).

yellow and oriental mustard, Sin a 1 and Bra j 1, both 2S albumins (Table 1), have been known for a long time [62,63]. Several IgE-binding proteins for sunflower seeds were identified, including a methionine-rich 2S albumin [64,65]. More recently, five allergens have been identified in sesame seeds [61^{••},66]. For three of these proteins, Ses i 1, Ses i 2, and Ses i 3, the full length amino acid sequences are available [67]. Interestingly, Ses i 1, Ses i 2, and Ses i 3 all belong to the family of seed storage proteins (Table 1). Looking at the entire sequence, the 2S albumin precursor of sesame seed (Ses i 2) has 38% homology to the walnut allergen Jug r 1, 40% homology to the Brazil nut allergen Ber e 1, and 34% homology to the peanut allergen Ara h 2 [61^{••}]. The 7S vicilin-type globulin of sesame (Ses i 3) has 36% homology to the peanut allergen Ara h 1 and 41% homology to the walnut allergen Jug r 2. As indicated above, one of the known IgE binding epitopes of Ara h 1 has a high degree of homology with the corresponding area of the sesame vicilin [61^{••}] (Fig. 3).

Fish

Parvalbumins have been identified as the major allergens in fish, with high cross-reactivity between different fish species. Parvalbumins are small proteins of about 12 kDa that are extremely resistant to heat and enzymes of the gastrointestinal tract. Gad c 1, the major allergen of Baltic cod, was purified and characterized more than 30 years ago [68,69]. This year, a new parvalbumin allergen of Atlantic cod, Gad m 1, encoded by a gene distinct from that of Gad c 1, has been identified [70]. The same group also reported a new minor allergen of codfish of

Figure 3. The amino acid sequences of several 7S globulins were aligned with Vector NTI Advance (InforMax, Bethesda, MD, USA)

(a)	321
Ses i 3 [sesame] (309)	LLQPVSTPG EFELFFGAGGENP
Ara h 1 [peanut] (288)	ISMPVNTPGQFEDFFPASSRDQ
Jug r 2 [walnut] (295)	LLQPVNNPGQFREYYAAGAKSP
Ana o 1 [cashew] (266)	FLRPVNLPGHFEVFGPGGENP
Cor a 11 [hazelnut] (175)	ILQPVSA PGHFEAFYGAGGEDP
(b)	411
Ses i 3 [sesame] (377)	SRHEEGG--IWPFGGESKG-TINIYQQR
Ara h 1 [peanut] (377)	TKHAKSVSKKGSEEEGDITNPINLREGE
Jug r 2 [walnut] (366)	SQHAMSAG-QRPWGRRSSGGPISLKSES
Ana o 1 [cashew] (334)	SRRGEGPK-IWPFTEESTG-SFKLFKKD
Cor a 11 [hazelnut] (243)	SQHEEGPPRIWPFGESESG-PINLLHKH

Shown are two regions as an example, with (a) high and (b) low homology and identity. Amino acids are shown as bold in gray boxes if they are identical in all aligned proteins and in bold only if they are identical in the majority of aligned sequences. Blocks of similar amino acids are shown in gray boxes and weekly similar ones as cursive. In addition, the known IgE-binding sites for Ara h 1 [39] and Ana o 1 [55[•]] in these regions are underlined. Amino acids critical for IgE binding for Ara h 1 are double underlined [83]. The IgE-binding region of Ara h 1 shown under (a) shows a high degree of homology especially to the sesame seed allergen Ses i 3 with all critical amino acids being identical [61^{••}]. The IgE-binding region of the cashew nut allergen Ana o 1 shown under (b) has no homology to the peanut allergen Ara h 1, but some to the hazelnut allergen Cor a 11 as well the to the sesame seed allergen Ses i 3; however, the critical amino acids for IgE-binding need to be identified to make further conclusions.

41 kDa that is homologue to an aldehyde phosphate dehydrogenase [71,72].

Recently, an IgE-reactive recombinant carp parvalbumin, rCyp c 1, mimicking the properties of the corresponding natural allergen, was obtained and characterized [73,74**]. This rCyp c 1 reacted with IgE from all fish-allergic patients tested, induced specific and dose-dependent histamine release, contained most of the IgE epitopes present in natural allergen extracts from cod, tuna and salmon, and might be useful in the diagnosis of fish allergy [74**].

Shellfish

The muscle protein tropomyosin has been identified as the major allergen in crustaceans, such as shrimp, crab, crawfish and lobster [75]. Tropomyosin consists of two parallel α -helical molecules that are wound around each other forming a dimer. Allergenic tropomyosin is found not only in crustaceans, but also in house dust mites and cockroach, suggesting tropomyosin as an important cross-sensitizing pan allergen [75]. In contrast, vertebrate tropomyosins are nonallergenic. A recent study identified the continuous, allergenic regions of the shrimp tropomyosin, Pen a 1, and compared these regions with homologous sequences in other allergenic and nonallergenic tropomyosins [76]. Five major IgE-binding regions, 15–38 amino acids long, could be identified. A high degree of sequence similarities between Pen a 1 IgE-binding regions and homologous regions in invertebrate tropomyosins, and a lower percentage of similarities with homologous regions of vertebrate tropomyosins were found, supporting a structural basis for cross-reactivity of allergenic tropomyosins [76]. Moreover, based on amino acid similarities and epitope reactivity, lobster tropomyosin has the greatest and cockroach tropomyosin the least cross-reactivity with shrimp [77*]. However, the clinical relevance of these cross-reactivities in developing allergic reactions to different arthropods needs to be determined. Very recently, a second shrimp allergen, Pen m 2, has been identified [78].

Wheat

IgE binding to water/salt-soluble and insoluble fractions of wheat has been found in children who experienced anaphylactic reactions after ingestion of wheat products, as well as in children with atopic dermatitis and wheat allergy [79]. Similarly, in adult patients suffering from gastrointestinal symptoms following wheat ingestion, IgE binding was found in soluble and insoluble wheat flour proteins [80]. The wheat α -amylase inhibitor has been identified as the relevant allergen in patients experiencing hypersensitivity reactions following the ingestion of wheat proteins as well as in bakers' asthma [81]. In addition,

it was shown that the majority of children with immediate allergic reactions to ingested wheat had IgE antibodies to ω -5 gliadin (Tri a 19) [14], which was previously identified as a major allergen in wheat-dependent, exercise-induced anaphylaxis [82].

Conclusion

Numerous allergens have been recently identified and characterized in a variety of foods including tree nuts and seeds. Most of these food allergens belong to few families of seed storage or pathogenesis-related proteins. These proteins can now be used in purified or recombinant forms for advanced in-vitro diagnostic approaches using protein microarray technology. In addition, previously identified food allergens have been further characterized in regard to their overall structure and IgE-binding sites. This characterization will be further accelerated through the development of peptide microarray technologies. Cross-reactivity between similar allergens from different species and its role in developing allergic reactions to different foods can now be studied more easily. These advanced measurements of specific food allergen or peptide-specific serum IgE concentration may enable us to determine more specific diagnostic decision points for predicting the outcome of oral food challenge tests and for predicting the natural history of the food allergic disorder.

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