

Improving diagnostic tests for food allergy with recombinant allergens

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Abstract

Food allergy is one important manifestation of atopic allergy. Primary food allergy mainly affects young children (class I), whereas adults frequently develop food allergy as a consequence of an inhalant sensitization (class II). At present, the diagnostic instrument for proving class II food allergy is not satisfactory. Skin tests as well as serological tests are in general neither very specific nor highly sensitive because they depend on food extracts, which differ in their content of individual allergens, vary between manufacturers, and even between different batches. Since the presence of food allergen-specific IgE antibodies does not always correlate with clinical symptoms against the respective food, oral provocation tests (ideally double-blind placebo-controlled food challenges) have to be performed to validate serological diagnosis or skin tests. However, oral provocation tests are connected to several practical problems and include a specific risk for the allergic patient. Applying DNA technology, up to 40 food allergens have been produced in recombinant form, which implies standardized quality and unlimited quantity of the respective proteins. Hence, such molecules might be used to solve problems of clinical and molecular allergology in diagnosis, research, and therapy of class II food allergies. First experiments with recombinant food allergens in this respect appear very promising.

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1. Introduction

The term “adverse reactions to foods” summarizes a wide range of symptoms caused by ingestion of certain foods and their ingredients, and a variety of different mechanisms underlie their clinical appearance. The presently valid position paper of the European Academy of Allergy and Clinical Immunology (EAACI) classifies these reactions according to the underlying pathomechanism in toxic and non-toxic reactions [1] (Fig. 1). Non-toxic adverse reactions to foods can be mediated immunologically. In this case, the term “food allergy” should be used. The most common form of food allergy is mediated by IgE antibodies and reflects an immediate type (“Type I hypersensitivity”) reaction, i.e., an acute onset of symptoms after ingestion. During sensitization,

specific IgE antibodies directed against food allergens are produced by the immune system and are capable of binding to high affinity receptors on the surface of mast cells and basophils. Cross-linking of the IgE antibodies upon re-exposure to the food constituents triggers the release of certain preformed mediators. The release of these mast cell mediators being stored in the mast cell granules, e.g., histamine, leads to the acute phase of the allergic immediate reaction. Mediators that are synthesized after mast cell activation, e.g., leukotrienes, prostaglandins, and cytokines, lead to recruitment of inflammatory cells causing typical inflammatory (“late-type”) reactions. On the other hand, allergenic food proteins are able to activate special subsets of T lymphocytes to home to the skin, and thus, aggravate atopic eczema [2,3].

Immediate reactions to food affect all age groups and involve multiple target organs. The clinical symptoms enclose localized symptoms at the site of direct contact

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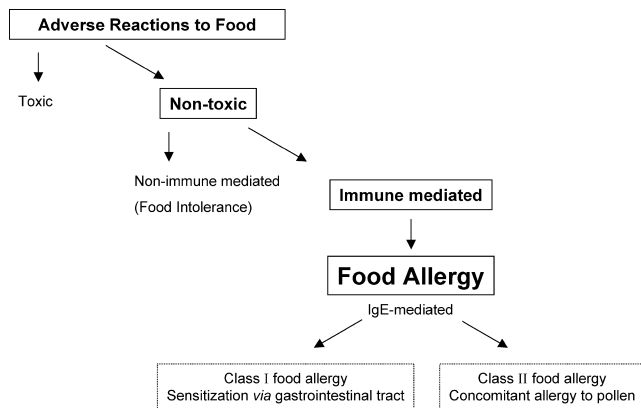


Fig. 1. Classification of adverse reactions to food.

(for example, at the mucosa of lips, tongue, and throat), contact urticaria (e.g., of the hands after contact with food proteins), and localized gastrointestinal allergy (nausea, cramping, pain, vomiting, flatulence, and diarrhea). Systemic symptoms occur in different organs after ingestion of food such as the skin (urticaria and angio-edema), respiratory tract (rhinitis and asthma), or the maximum variant of an immediate type allergic reaction (anaphylaxis with cardiovascular and gastrointestinal symptoms sometimes leading to shock).

According to the clinical appearance, the pattern of allergens, and the underlying immunological mechanisms, two different forms of IgE-mediated food allergy have to be distinguished (Fig. 1). In class I food allergy, the sensitization process occurs via the gastrointestinal tract. This type of food allergy can be the first manifestation of the atopic syndrome and mainly affects young children [4]. The most important allergens are cow's milk, hen's egg, and legumes causing or, in addition or instead of immediate type reactions, influencing atopic dermatitis in many cases [5]. With some exceptions (peanut and fish), these manifestations disappear later during childhood in the majority of the cases, but will be replaced by other manifestations of the atopic syndrome [6].

The second type (class II) of food allergy develops later in life and mainly affects adolescents and adults. This disease is believed to develop as a consequence of an allergic sensitization to inhalant allergens and the basis for this food allergy is immunological cross-reactivity, which is due to a high amino acid sequence identity and structural homology between food and pollen allergens. Patients with birch pollen allergy, for example, may develop symptoms after the ingestion of fresh apples, hazelnuts, raw carrots or celery [7–11]. Several proteins homologous to minor birch pollen allergens were detected in these birch pollen-related foods, such as profilins and isoflavone reductase-like proteins [12,13]. More importantly, the major allergen in birch pollen, Bet v 1, belongs to the pathogenesis-related

(PR)-10 protein family, which also comprises major allergens of several birch pollen-related fruits and vegetables [14–21]. In most of these cases, the “oral allergy syndrome” (OAS) is the prominent clinical symptom. Recently, it was reported that a member of the PR-10 protein family from soybean, SAM 22, was capable of inducing severe allergic reactions [22]. Furthermore, anaphylactic shock reactions are not rare in the mugwort-birch-celery-syndrome [11,23,24]. Thermostable allergens of higher molecular weight might seem responsible for these reactions [11,25]. Obviously due to the steadily increasing prevalence of inhalant allergies, class II food allergy found increasing attention. However, this kind of food allergy is especially difficult to diagnose. This is mainly due to the fact that, in general, commercially available food extract preparations are not suitable for the diagnostic tests. The major allergens involved in this type of food allergy are particularly susceptible to degradation processes caused by constituents of the food matrix and are thus easily destroyed during the extraction procedure. Recombinant DNA technologies allowed to produce several of these allergens in a pure and stable form. Therefore, recombinant food allergens could contribute to improve the diagnosis of food allergy. In addition, the use of these molecules facilitates an important progress from an extract-based diagnosis to a component-resolved diagnosis which may result in refined information about cross-reactivity patterns and potential severity of the symptoms.

2. Diagnosis of food allergy

The diagnostic approach to suspected adverse food reactions begins with a careful case history and clinical examination. When IgE-mediated food-induced allergic reactions are suspected, *in vivo* skin tests and *in vitro* serological assays are useful to evaluate whether the patient possesses IgE antibodies to certain foods. For practical reasons, skin prick tests (SPT) represent the method of choice to demonstrate the sensitization to defined food antigens in a patient *in vivo*. The test solution containing the allergen is applied to the skin, which is subsequently pricked with a lancet with 1 mm tip [26]. The appearance of wheals caused by mediator release of IgE-sensitized cells is subsequently recorded.

Serological assays, such as radio-allergosorbent test (RAST), enzyme-allergosorbent test (EAST), enzyme-linked-immunosorbent assay (ELISA) or immunoblotting, evaluate allergen-specific IgE antibodies in the serum of the allergic patient. Food extracts are immobilized onto surfaces (tubes, plates, cellulose, and nitrocellulose) and after incubation with serum, bound IgE is detected either by radioactive- or enzyme-labeled anti-human IgE antibodies. Immunoblotting offers the great advantage that IgE-binding to individual proteins

can be analyzed [27,28]. The total allergen extract is first subjected to a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), leading to a separation of all proteins according to their molecular size [29]. The separated proteins are subsequently transferred onto a nitrocellulose, which represents a more solid carrier than the polyacrylamide gel. IgE binding patterns of different patients can be determined and according to the frequency of IgE antibodies bound to particular proteins, major or minor allergenic components of allergen sources can easily be determined. Furthermore, an allergen might be detected even when present in the extract only at low concentrations. However, this methodology requires a particular laboratory equipment and is therefore not appropriate for routine diagnosis.

Since serological tests and SPT solely indicate the presence of allergen-specific IgE but do not diagnose the clinical appearance of food allergy, oral provocation tests or diagnostic diets have to be performed in certain cases to confirm or to refute the patient's food allergy. Although several oral provocation models exist, at present, the double-blind placebo-controlled food challenge (DBPCFC)¹ is considered as the “gold standard” for diagnosis of adverse reactions to food [1,30,31]. The food under investigation is given to the patient via a masked vehicle to blind its taste and texture. Hence, the patient's subjective bias is minimized. Provided it is performed properly, a positive DBPCFC is still the only conclusive evidence of a food allergy.

3. Diagnostic problems concerning class II food allergy

The diagnostic approach presented above is conclusive in most cases of class I food allergy and provides useful information for the allergologist about the specificity and quantity of the respective sensitization. However, this is not the case for the diagnosis of class II food allergy. Most allergens involved in this type of food allergy are plant-derived proteins, which are easily degraded by physical and/or chemical strain during the extraction process. This does not only result in difficulties during the preparation of food extracts but also implies that certain allergens within an extract may be degraded during the storage for longer time periods. On the one hand, the final amount of an allergen in an extract depends on the raw material used and the methodology applied to extract the proteins. For instance, an optimal content of lipid-transfer proteins (PR-14 protein family) is achieved when fruit peel is used as source for extraction [32,33]. However, using this approach, allergens belonging to the PR-10 family and profilins will be

under-represented in the final extract, since they are located mainly in the flesh of fruits. The same problem applies to pH conditions during the extraction process. Whereas optimal amounts of PR-14 family proteins are obtained under slightly acidic conditions, members of the PR-10 protein family are ideally extracted at pH 8.6. On the other hand, different plant strains may contain different amounts of allergens as has been demonstrated for apples [27]. Finally, many allergens inducing class II food allergy are susceptible to degradation by inherent enzymatic activities [34,35]. The enzymes are released during the mechanical crushing of the food and certain allergens might be degraded even before the actual extraction begins. Furthermore, the degradation might continue during longer periods of storage, which continuously changes the composition of the initial food extract. Hence, the standardization of food extracts according to total protein content, content of single allergens or allergenic activity is in many cases unfeasible. Several attempts to improve this situation have been successfully performed [36,37]. Unfortunately, these approaches require complex extraction procedures and are therefore not suited to prepare food extracts routinely.

The sensitivity and reliability of SPT and serological tests mainly depend on the quality of food extracts. Although the negative predictive values were shown to be excellent, positive predictive accuracies of SPT are not very high in class II food allergy [38]. Using fresh food instead of food extracts (prick-to-prick technique) increases the sensitivity of skin tests [39]. Nevertheless, this again relies on an allergenic source, which is not characterized or standardized in anyway.

Besides all the difficulties to obtain a reliable serological diagnosis of class II food allergy, also oral provocation tests may give questionable results. In this respect, the diagnosis of OAS is particularly difficult to handle, since the food should not be swallowed immediately but kept in the mouth for a certain time. Due to the “instability” of most class II food allergens and their rapid degradation by endogenous enzymes in the food, truly allergic patients may show a negative DBPCFC to the active probe but react positively to an open challenge with the fresh food. Furthermore, different challenge models might differ in both, the sensitivity and the number of placebo reactions. For example, DBPCFC to diagnose apple allergy carried out with either apple juice or grated apples in the active probes significantly varied in their sensitivity [40]. Additional difficulties result from finding suited ingredients, which blind the food under evaluation in the active meal in order to minimize the patient's subjective bias. To verify allergy to celery, carrot, and cherry, a two-step “spit and swallow” protocol has been developed, which not only considers the specific clinical features of OAS but also increases the safety of the patients [41–43]. Nevertheless, the need for

¹ Abbreviations: DBPCFC, double-blind placebo-controlled food challenge; SPT, skin prick tests.

standardized challenge models and suited recipes for challenge and placebo preparations remains [31].

Taken together, the diagnostic instruments for class II food allergies are not sufficient at present. One approach to solve this problem might be the use of recombinant food allergens. On the one hand, these proteins may be applied to standardize food extracts (discussed in this issue by Ronald Van Ree). On the other hand, one single allergen molecule or a panel of a few recombinant allergens might be suited to replace allergen extracts in the diagnosis of certain food allergies. Hence, the employment of standardized and well-characterized recombinant allergens could improve the sensitivity, specificity, and reproducibility of diagnostic tests in food allergy.

4. Recombinant food allergens

The application of recombinant DNA technology to synthesize food allergens has greatly enhanced our knowledge about their structure and function. Up to now more than 40 recombinant food allergens have been produced. Table 1 provides a currently valid summary of major and minor recombinant food allergens of animal or plant origin. Several recombinant allergens involved in class II food allergies were tested for their suitability for *in vivo* and/or *in vitro* diagnosis. The initial experience with these allergens as diagnostic tools is very encouraging. Recombinant Api g 1, the major allergen of celery, was used for skin tests. The results indicated that this protein allowed accurate *in vivo* diagnosis of celery allergy in areas where birch trees are common [44,45]. In the case of hazelnut allergy, 43 sera of patients with DBPCFC-proven allergy to hazelnuts were serologically investigated either with hazelnut extract or with the recombinant major allergen Cor a 1.0401 [21]. EAST with recombinant Cor a 1.0401 yielded a sensitivity of 95% compared to 70% of the commercially available CAP/RAST system, which is based on a total food extract. Very recently, taking cherry as a model food, a panel of three recombinant allergens (Pru av 1, Pru av 3, and Pru av 4) was tested for its capability to diagnose cherry allergy by SPT in comparison to DBPCFC [43]. The commercially available cherry extract produced a positive skin prick response in 20% whereas the panel of recombinant proteins reached a sensitivity of 96%. Furthermore, EAST using recombinant Pru av 1 and Pru av 4 revealed 97% positive results in 101 cherry allergic patients in comparison to only 17% by CAP/RAST [33]. Taken together, these data strongly support the concept that recombinant food allergens are suited molecules to replace food extracts in the future. However, positive serological test results and SPT do not necessarily reflect a clinically relevant food allergy. The existence of allergen-specific

IgE does not inevitably correlate with symptoms when eating the respective food [34,46,47]. The use of recombinant allergens will not overcome this problem. Seventy percent of birch pollen allergic patients who tolerated cherry in an open challenge test reacted in SPT with recombinant allergens from cherry [43]. Similarly, sera from patients with a positive and negative DBPCFC to celery root showed IgE binding to recombinant Api g 1 [24]. Although recombinant food allergens improve the reliability and accuracy of the diagnostic material for SPT and serological assays, to confirm the clinical symptoms against certain foods one cannot surpass the DBPCFC. Nevertheless, recombinant food allergens might also be very usefully applied for oral provocations. Being without taste and applicable at a defined concentration, the blinding and standardization of the challenge probes could easily be managed. Hence, any subjective impressions by the patients could be definitively excluded. In addition, the development of standardized protocols for oral provocation tests would certainly be simplified. Finally, the risk to induce concomitant undesired or even dangerous side effects might be reduced.

5. Concluding remarks

Besides the benefits for the diagnosis of food allergy including both, *in vitro* and *in vivo* testing, allergens in recombinant form will help us to understand basic immunological mechanisms operative in class II food allergy. Purified recombinant proteins have successfully been used to identify cross-reactive B cell epitopes in related pollen and food allergens such as profilins, isoflavone reductase-like proteins or proteins of the PR-10 family [13,18,19,32,48]. In a first study, cross-reactive T cell epitopes of Bet v 1 and Mal d 1 were analyzed, and the central role of allergen-specific T helper class 2 lymphocytes in the pathogenesis of IgE-mediated class II food allergy was confirmed as has been shown for pollen-induced allergy in many studies [49]. Studies like these will contribute to define whether pollen or food allergens represent the initial sensitization in class II food allergy and to enlighten the potential role of food allergens for the typical maintenance of pollen-specific IgE titers outside the pollen season.

In summary, recombinant food allergens have increased the knowledge about chemical and immunological features of these proteins and allowed first insights into immunological mechanisms underlying class II food allergies. Based on this, novel strategies for the treatment of food allergies may be developed. Allergens can be modified to lose their IgE-binding capacities but to retain T cell stimulating activities. The resulting proteins are termed “hypoallergens” and represent candidate molecules for a safe specific

Table 1
Recombinant food allergens

Recombinant food allergen (*)	Source	Function	Characterized by	Reference
Ana o 1	Cashew	<i>Vicilin-like seed storage protein</i>		[52]
Api g 1	Celery	<i>PR-10 protein</i>	IgE immunoblot, compared to extract, IgE immunoblot inhibition, EAST, RAST, EAST inhibition, SPT, mediator release	[15,45,48,53]
[Api g 2]	Celery	<i>PR protein</i>	IgE binding reported, but data not shown	[54]
[Api g 3]	Celery	<i>Chlorophyll alb binding protein</i>	No immunological characterization	[54]
Api g 4	Celery	<i>Profilin</i>	IgE immunoblot, IgE immunoblot inhibition, EAST, reported but data not shown, EAST inhibition, mediator release	[48]
Ara h 1 (68 kDa)	Peanut	<i>Vicilin-like seed storage protein</i>	IgE immunoblot, compared to nAra h 1	[55]
Ara h 1 (48 kDa)	Peanut	<i>Conglutin seed storage protein</i>	IgE immunoblot, compared to nAra h 1	[56]
Ara h 2	Peanut		IgE immunoblot, T cell proliferation	[57,58]
Ara h 3	Peanut	<i>Legumin-like 11S seed storage protein</i>	IgE immunoblot	[59]
Ara h 4	Peanut	<i>Glycinin</i>	IgE immunoblot	[56]
Ara h 5	Peanut	<i>Profilin</i>	IgE immunoblot	[56]
Ara h 6	Peanut	<i>Conglutin-like seed storage protein</i>	IgE immunoblot	[56]
Ara h 7	Peanut	<i>Conglutin-like seed storage protein</i>	IgE immunoblot	[56]
Bos d 5 [bovine β -lactoglobulin]	Cows' milk	<i>Retinol binding protein</i>	Immunoblot with two monoclonal antibodies, immunoblot with polyclonal rabbit anti-serum, ELISA, compared to nBos d 5	[60–62]
[Cha f 1]	Crab	<i>Tropomyosin</i>	IgE immunoblot, IgE immunoblot inhibition	[63]
[Cha n ?]	Scallop	<i>Tropomyosin</i>	IgE immunoblot and inhibition studies reported, but data not shown	[64] MA
Cor a 1.0401	Hazelnut	<i>PR-10 protein</i>	IgE immunoblot, EAST, compared to extract, mediator release compared to extract reported, but data not shown	[21]
Cor a 9	Hazelnut	<i>11S globulin seed storage protein</i>	IgE immunoblot reported, but data not shown	[65]
Cra g 1.03	Oyster	<i>Tropomyosin</i>	IgE immunoblot inhibition	[66]
[Cyp c 1.01]	Carp	<i>Parvalbumin</i>	IgE immunoblot, mediator release	[67–69]
Dau c 1	Carrot	<i>PR-10 protein</i>	IgE immunoblot, compared to extract, IgE immunoblot inhibition	[18]
Gly m 1	Soybean	<i>Hydrophobic protein of soybean</i>	IgE binding reported, but data not shown, type of assay not mentioned	[70] MA
Gly m 3	Soybean	<i>Profilin</i>	IgE immunoblot, IgE immunoblot inhibition, EAST inhibition, IgE immunoblot	[71]
[Gly m ? (two glycinin acidic chains)]	Soybean	<i>Glycinin</i>	IgE binding reported, but data not shown, type of assay not mentioned	[70] MA
[Hal d ?]	Abalone		IgE immunoblot and inhibition studies reported, but data not shown	[64] MA
[Hom a 1]	Lobster	<i>Tropomyosin</i>	IgE immunoblot, IgE immunoblot inhibition	[63]
Jug r 1	Walnut	<i>2S albumin seed storage protein</i>	IgE immunoblot, FEIA inhibition	[72]
Jug r 2	Walnut	<i>Vicilin-like seed storage protein</i>	IgE immunoblot, IgE immunoblot inhibition	[73]
[Jug r (19 kDa)]	Walnut	<i>2S albumin seed storage protein?</i>	IgE binding reported, but data not shown, type of assay not mentioned	[74] MA
Mal d 1	Apple	<i>PR-10 protein</i>	IgE immunoblot, compared to extract, IgE immunoblot inhibition, T cell proliferation assay, cytokine assay	[16,49]
Mal d 1 (three isoforms)	Apple		IgE immunoblot, compared to nMal d 1, EAST, compared to nMal d 1, mediator release	[8]
Met e 1	Shrimp	<i>Tropomyosin</i>	IgE immunoblot, IgE immunoblot inhibition reported, but data not shown,	[63]
Ory s 1	Rice		IgE immunoblot inhibition reported, but data not shown,	[75]

Table 1 (continued)

Recombinant food allergen (*)	Source	Function	Characterized by	Reference
[Pan s 1]	Lobster		IgE immunoblot, IgE immunoblot inhibition	[63]
Pen a 1	Shrimp	<i>Tropomyosin</i>	IgE immunoblot, compared to nPen a 1	[76]
[Per v ?]	Mussel	<i>Tropomyosin</i>	IgE immunoblot and inhibition studies reported but data not shown	[64] MA
Pers a 1 [Prs a 1]	Avocado	<i>Endochitinase</i>	IgE immunoblot, IgE immunoblot inhibition	[77]
Pru av 1	Cherry	<i>PR-10 protein</i>	IgE immunoblot, IgE immunoblot inhibition, EAST, mediator release	[17,19]
[Pru av 1]				
Pru av 4 [Pru a 4]	Cherry	<i>Profilin</i>	IgE immunoblot reported, but data not shown, EAST, mediator release reported, but data not shown	[32,78]
Pyr c 1	Pear	<i>PR-10 protein</i>	IgE immunoblot, IgE immunoblot inhibition, EAST, compared to extract, mediator release	[19,20]
Pyr c 4	Pear	<i>Profilin</i>	IgE immunoblot, IgE immunoblot inhibition, EAST, compared to extract, EAST inhibition, mediator release	[32]
Pyr c 5	Pear	<i>Isoflavone reductase-like protein</i>	IgE immunoblot, IgE immunoblot inhibition, EAST, compared to extract, mediator release	[13]
Sal s 1	Salmon	<i>Parvalbumin</i>	IgE immunoblot, ELISA	[79]
SAM 22	Soybean	<i>PR-10 protein</i>	IgE immunoblot, EAST,	[22]
Sin a 1	Mustard	<i>2S albumin seed storage proteins</i>	IgE immunoblot, ELISA inhibition, polyclonal rabbit anti-Sin a 1-serum	[80,81]
[wheat glutenins and gliadins]	Wheat	<i>Glutenin</i>	IgE immuno dotblot	[82]

(*) Food allergen is not noted in the official WHO/IUIS list of allergens or is denominated differently by the authors in the cited reference.

MA, references are only meeting abstracts.

PR, pathogenesis-related.

kDa, kilo Dalton.

immunotherapy of allergic patients. In this respect, very promising approaches were presented for the major birch pollen allergen, Bet v 1 [50,51]. Besides improving diagnosis, improving therapeutical possibilities remains another important aim for the future, since at present the avoidance of the respective food is the only way to evade IgE-mediated reactions in allergic patients. Hence, recombinant food allergens might answer many questions of both, immunologists as well as allergologists, of which finally and most importantly the allergic patient will profit.

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