Review

Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel foods

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The introduction of novel proteins into foods carries a risk of eliciting allergic reactions in individuals sensitive to the introduced protein and a risk of sensitizing susceptible individuals. No single predictive test exists to perform a hazard assessment in relation to allergenic properties of newly expressed proteins in gene-modified organisms (GMOs). Instead, performance of a weighted risk analysis based on the decision tree approach has been suggested. The individual steps of this analysis comprise sequence homology to known allergens, specific or targeted serum screens for immunoglobulin E (IgE) cross-reactions to known allergens, digestability studies of the proteins in simulated gastric and/or intestinal fluids, and animal studies. These steps are discussed and five examples of risk evaluation of GMOs or novel foods are presented. These include ice-structuring protein derived from fish, microbial transglutaminase, GMO-soybeans, amylase and the Nangai nut.

Keywords: Food allergy / Genetically modified organisms / Radioallergosorbent test

1 Introduction

The pathogenesis of immunoglobulin E (IgE)-mediated food allergies can be divided into an afferent sensitization phase where the immune system develops an IgE response to one or more constituents (allergens) of a food, and an efferent eliciting phase where a clinical allergic reaction occurs after ingestion of the food in question. Accordingly, the term allergenic may be understood both as the capacity to sensitize, i.e., induce an IgE immune response, and as the capacity to elicit an allergic reaction in an already sensitized individual. Our understanding of the process leading to sensitization of a patient is still immature, but both factors intrinsic (dose, primary to quaternary structure defining what the immune system recognizes as epitopes) and extrinsic (adjuvant effect, host specific factors) to the individual molecule are involved. Only a limited literature is available on models testing the allergenicity – understood as ability to sensitize humans – of molecules or mixtures. The other meaning of allergenic, i.e., the eliciting of an allergic reaction in an already sensitized individual, has been much more successfully investigated and is described below.

2 Strategies

2.1 Strategies for identifying allergens

An allergen is an antigen to which IgE-antibodies will bind. In order to be functional there should be at least two – not...
necessarily different – epitopes in order to cross-link IgE when this is bound to its high affinity receptor, FceRI, on the surface of effector cells: mast cells or basophil granulocytes. The knowledge that biological materials, be they foods or airborne matter such as pollen, induce allergies goes back to the last part of the 19th century (pollen as the causative agent of hayfever identified by Wyman and Blackley in the 1870s) or even before in the case of foods causing allergies [1]. In the beginning of the 20th century, Prausnitz and Küstner [2] convincingly demonstrated that the sensitivity to codfish was transferrable via a serum factor, much later to be identified as IgE [3, 4]. With the advent of IgE and anti-IgE antibodies, molecular methods became available to characterize individual allergens by radioimmunoassays [5] and electrophoretic methods [6]. The first allergen to be sequenced was allergen M from codfish (Gad c1) [7], and during the last 30 years a large number of native and recombinant allergen molecule sequences have become available.

However, the definition that an allergen is an IgE-binding (-cross-linking) molecule does not per se imply that a particular protein can elicit allergic reactions in food-allergic patients. It is well-known that IgE-binding to food proteins may occur in persons that are clinically tolerant to the food from which they are derived, and this poses great challenges in developing better and more valid diagnostic reagents for food-allergic patients. In order to better define such clinically relevant allergens, the strategy has been to identify patients with a clinically proven allergy to a food item, i.e., to challenge patients in a double-blinded, placebo-controlled food challenge [8]. Serum samples from such patients have been used for characterization of allergens by testing the IgE-binding to extracts of the food, e.g., hazelnuts [9] or cloned proteins [10]. In the near future IgE-binding to cloned libraries of food proteins may become an option (Vieths and Cramer, personal communication). It must be emphasized, however, that even if a careful selection has been performed of patient sera for such studies, the definition of these proteins as clinically relevant allergens, still relies on the assumption that all proteins to which IgE has been developed in food-allergic patients are, in fact, relevant to the disease. A formal proof of this assumption would call for clinical challenges of food-allergic patients using purified or recombinant allergens – an enormous endeavour requiring large amounts of clinical research. So far there has been only limited interest in performing such studies since, for clinical reasons, it is sufficient for the patient to know that he or she is allergic to a particular food. However, when genes and proteins can be transferred freely from one biological material to another, there may be an increased interest in knowing whether an IgE-binding protein is, in fact, a clinically relevant allergen. Such knowledge may also be of importance if strategies to develop low-allergenic foods based on eradication or reduced expression of certain genes should be successful.

As discussed below, the number of known allergenic protein sequences probably amounts to more than 2500, and the figure is constantly growing. Since many isoforms exist within each species and there is a large degree of cross-reactivity between botanically or zoologically related species, it is likely that there is a bias in the allergen databases towards sequences with homology to previously discovered and described proteins. Thus, the discovery of a given protein such as the pathogenesis related group 10-proteins (birch pollen Bet v1-homologues) [11], or the lipid transfer proteins in fruits and nuts [12], will spur the search for homologous proteins in a large number of species, without necessarily identifying the most important or relevant allergens for these species.

2.2 Strategies for predicting allergens in gene-modified organisms

The introduction of novel proteins into foods carries a risk of eliciting allergic reactions in individuals sensitive to the introduced protein or of sensitizing susceptible individuals (e.g., ato(p)ics). No single predictive test can identify the allergenic potential of an unknown protein, but various schemes have been formulated. An early attempt was the IFBC/ILSI Decision Tree for the assessment of the allergenicity of foods produced by genetic modification [13]. This scheme was subsequently adopted by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) and extensively modified and updated in an expert consultation held in 2001 [14]. Each of these decision trees identifies features of allergens which must be considered and tests which should be performed to give assurance that a novel protein is not likely to elicit reactions. These schemes thus use a weight of evidence approach to assess the likelihood that a novel protein will prove allergenic.

The FAO/WHO Decision Tree starts by analyzing the primary amino acid sequence of the novel protein and looking for similarity with known allergens. This is followed by in vitro investigations with sera from patients with an established allergy to the source of the protein whenever possible. If such tests are negative, and especially when only few sera from confirmed allergic patients are available, this procedure can be followed by targeted serum screening using sera from patients with allergies to foods of a related type. Since resistance to proteolytic degradation has been described as a characteristic feature of several allergenic proteins, a standardised protocol for investigation of this property is also included in the decision tree.

In the present review the FAO/WHO Decision Tree is partly being built upon. Recently, a meeting of Codex Alimentarius suggested taking a more liberal standpoint to the individual branches of the decision tree, but this has been much
criticized and, for a producer of a GMO, it would be prudent to try to fulfill the FAO/WHO guidelines since these are the most specific and updated guidelines published so far. Thus far, the decision tree is purely qualitative, providing a Yes/No answer to the question: “Should a new protein be considered as an allergen?” A more detailed description defines the homology to more than 35% over a window of 80 amino acids or an identity of at least six contiguous amino acids over the entire sequence [14]. This algorithm has been criticized for including too many proteins, since it was estimated that 67% of all protein sequences in the Swiss-Prot database would be identified as potential allergens. Reducing the stringency by evaluating 7, 8, or 12 contiguous residues reduced the percentages to 17.6%, 8.7%, and 7.2%, respectively. Moreover, 43% of the sequences in a database derived from the human genome were identified as potential allergens [15]. Other investigators have found the same tendency using a smaller number of test sequences [16, 17].

In comparison, by introduction of a motif-based algorithm based on known allergens it was possible to reduce the number of positive hits in Swiss-Prot to 2–4% [15] allegedly without losing sensitivity. Various suggestions for improvements have been suggested including use of a two-step alignment procedure, not only taking into account full identity between a stretch of the sequence, but also – in a second step – scoring for conservative replacements that would maintain the overall structure of the entire region [18]. Likewise, addition of general antigenicity algorithms as a second step has additionally been suggested [17], which is supposed to reduce the number of unspecific candidates, i.e., increase the specificity of the screening without losing sensitivity.

When homology comparisons are made, the database of the primary sequence of known allergens becomes highly important. Gendel [18] has described some of the problems in using general protein databases, and creation of dedicated allergen databases is generally recommended. There exist a number of nonredundant allergen databases in the public domain (reviewed in [19], to which should be added www.allergome.org), and when an attempt was made to merge these in September 2001, 2643 unique protein sequences resulted. During the process a significant number of errors was found, and clearly more work is needed to create a public allergen database with high validity.

It should be mentioned that strategies which only look at the primary sequence may seriously fail to detect conformational epitopes which are composed of a small number of amino acid residues from noncontiguous parts of the primary sequence. With the advent of the solutions of 3-D structures of allergens [20], it has become increasingly clear that both IgG- and IgE-binding epitopes [21, 22] may be of the conformational type and that disruption of the tertiary

3 Homology between a novel protein and known allergens

The FAO/WHO Decision Tree suggests procedures for investigating homology between a new protein and proteins already known as allergens. “The first step is a database search for an allergen with a homologous amino acid sequence, according to the principles described in Section 6.1. If this search reveals a level of homology with a known allergen that suggests a potential for cross-reactivity, the expressed protein is considered to be an allergenic risk. No further evaluation for allergenicity would typically be necessary.” A more detailed description defines the homology to more than 35% over a window of 80 amino acids or an identity of at least six contiguous amino acids over the entire sequence [14]. This algorithm has been criticized for including too many proteins, since it was estimated that 67% of all protein sequences in the Swiss-Prot database would be identified as potential allergens. Reducing the stringency by evaluating 7, 8, or 12 contiguous residues reduced the percentages to 17.6%, 8.7%, and 7.2%, respectively. Moreover, 43% of the sequences in a database derived from the human genome were identified as potential allergens [15]. Other investigators have found the same tendency using a smaller number of test sequences [16, 17].

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structure can have dramatic consequences for these important epitopes [23, 24].

4 Specific or targeted serum screening

The FAO/WHO Decision Tree next suggests procedures for screening with relevant source organisms: “Specific serum screening for the expressed protein focus on assessment of the possible allergenicity of the expressed protein using sera from patients allergic to the source material (Section 6.2). If a positive response is found, the expressed protein should be considered allergenic. If no such sera exists or none are found positive, cross-reactivity may be tested with a panel of serum samples that contain high levels of IgE antibodies with a specificity that is broadly related to the gene source (Section 6.3). For this “targeted serum screen”, 6 groups of source organisms are distinguished: yeast/ moulds, monocytes, dicots, invertebrates, vertebrates and “others”. A panel of 50 serum samples with high levels of IgE to allergens in the relevant group is used to search for IgE antibodies that are cross-reactive with the expressed protein. If a positive reaction is obtained with one of these sera, the expressed protein is considered to be an allergenic risk and further evaluation for allergenicity would typically not be necessary. If a gene were obtained from a bacterial source, no targeted serum screening would be possible, since no normal population of individuals are known to be sensitised (IgE-mediated) to bacterial proteins.”

4.1 IgE-reactivity to allergen extracts

(RAST-type methods)

Since IgE was first purified and anti-IgE antisera became available [3, 25–27], immunoassays have been designed for specific IgE using either radiolabelled allergen [28, 29] or radiolabelled anti-IgE antibody as in the radioallergosorbent test (RAST) [30]. The initial design of RAST was based on the use of dextran-derived materials [28, 30], but later solid phases comprised of widely used paper discs [31], aluminum hydroxide gel [32], polystyrene tubes [33], cellulose polymers [34, 35], and magnetic microparticles [36] have gained popular use. Alternative to varying the solid-phase, the detection principle has also been modified comprising enzyme catalyzed reactions (enzyme-linked allergosorbent tests, EASTs), fluorescence and chemilumino-nometric procedures. Reviews of the available technologies and a discussion of method evaluation are given in [37, 38].

It is possible to use RAST-type methods either directly or indirectly, i.e., as inhibition RAST, to detect IgE-binding to an allergen. In the direct method, the extract is coated on the solid phase which makes it necessary to check that the relevant component is actually present in sufficient amounts and that it is bound properly to the solid-phase. In the inhibition RAST, even small amounts of a component in a complex mixture may be detected provided that the response of the basic system, i.e., the direct RAST that is to be inhibited, represents the relevant binding between the component and the specific IgE.

4.2 Immunoblotting and comparable methods

A qualitative description of the IgE-binding antigens in a mixture may be obtained by subjecting the allergen extract to primary separation. Such analysis is based on molecular weight (SDS-PAGE), isoelectric point (isoelectric focusing) or both (2-D gels), or combinations of physicochemical and immunochemical characteristics (crossed immunoelectrophoresis, CIE) as separation principles. After fixation of the separated pattern of components, the IgE-binding antigens may be detected by secondary immunodetection involving incubation with antibody followed by a labelled anti-IgE antibody. Examples of such methods are Western blotting on nitrocellulose membranes transferred from SDS-PAGE molecular weight separations or the crossed immunoelectrophoresis (CIE) method, in which case an IgE-immunoradiometric assay is performed in overlay on the pattern of precipitates in a CIE-gel. A comparative study has suggested that the ideal result is only obtained by a combination of the different methods [39].

4.3 Passive sensitization of human basophils

The principle of the method is to challenge sensitized basophils with allergen which will cross-link surface-bound specific IgE causing histamine to be released from the cells. Histamine is determined and a dose-response curve can be constructed and be compared with an appropriate standard. In order to ensure that the basophils are responding properly, the universal reagent anti-IgE is applied as a positive control, whereas the test substance is applied to basophils with no specific IgE to ensure that no nonspecific histamine release, i.e., release caused by cytotoxicity, takes places. Histamine can be measured fluorometrically after coupling to a fluorophore (o-phthaldialdehyde). Alternative methods for detection of histamine are immunochemical: the immuno-tech radioimmunoassay [40] and the automated fluorometric histamine assay [41]. Agreement between the three methods has been established [42, 43].

In one application of the histamine release method, glassfiber-coated microtiter plates are used for separation of histamine from other constituents in the assay [44, 45]. Heparinized, washed blood may be used without further separation, but the use of whole blood or gradient-enriched basophil suspensions have also been described. Dilutions (25:1)
of blood cells are stimulated in glassfiber-coated microtiter plates with dilutions of the test protein for 1 h at 37°C. Histamine is subsequently separated from the cellular suspension since it is absorbed to the glassfibers of the microtiter plate. The plates are washed extensively, and the histamine can be released by a change in pH and finally measured fluorometrically after coupling to a fluorophore (o-phthaldialdehyde). The histamine determination is calibrated by use of histamine standards in the medium, which are incubated in glassfiber wells in parallel with the blood samples. A number of biogenic amines (spermidine, cadaverine), histamine metabolites and histidine have been tested and found not to interfere with the assay [45]. Since some allergen extracts have been reported to contain histamine [46] it is essential that the histamine content of each preparation of test protein is assessed. Moreover, since new and unproven preparations of allergens or other offending substances may have cytotoxic activities, a control should always be carried out using a nonallergic basophil donor. It is possible to strip IgE from basophils and sensitize the cells with IgE from another donor by incubating serum together with the stripped basophils, i.e., a passive sensitization. In this way it is possible to use basophils from normal donors together with stored serum samples from patients with the relevant specific IgE [47].

4.4 Selection of sera for serum screening

The guidelines underscore the importance of having strongly positive sera from patients with a well-documented food allergy to the relevant source. Rather than testing many sera with low specificity and/or low titers, it is important to employ high titer sera from patients evaluated according to international guidelines, such as the newly published European Academy of Allergology and Clinical Immunology guidelines for double-blinded, placebo-controlled food challenges [48]. If the protein in question does not constitute a major allergen (i.e., an allergen to which more than 50% of an allergic population reacts), a large number of sera may be necessary to achieve sufficient certainty that an allergenic protein may be identified, i.e., 24 sera will assure that an allergen to which more than 20% of the population react will be detected with 99% certainty [14].

5 Digestability and animal models

Finally, the FAO/WHO Decision Tree suggests procedures for screening for digestability and immunogenicity: “If no cross-reactive serum is found, the protein is analysed for pepsin resistance and for evidence of immunogenicity in appropriate animal models according to the protocols provided in Sections 6.4 and 6.5.” If neither homology screening nor specific or targeted screens identify allergenic properties for a newly expressed protein, further evaluation by pepsin digestability should be invoked since it was demonstrated that resistance to digestion by pepsin correlated with allergenicity [49]. Later work, however, cast some doubt on the usefulness of this test since few of all known food allergens demonstrate resistance to simulated gastric fluid (SGF-containing pepsin) or to simulated intestinal fluid (SIF) comprising pancreatin (a mixture of five enzymes: amylase, trypsin, lipase, ribonuclease, and protease) [50]. An explanation for the lack of correlation between SGF-digestability and nonallergenicity may be that both children and adults may have naturally or iatrogenically increased ventricular pH for extended periods [51]. Moreover, the applied protocols seem to have profound influence on the results, but by using identical protocols a good inter-laboratory agreement has been obtained [52].

A number of interesting animal models of food allergy have been developed, and mice [53], rats [54], guinea pigs, dogs, and swine have been employed as experimental animals, reviewed in [55]. Thus far, however, no consensus regarding a particular species or an immunization protocol has been reached.

6 Cases

To demonstrate the applicability of the methods discussed above, five cases of risk evaluation are described below. Whereas the FAO/WHO consultation was aimed at “Foods derived from biotechnology” which translated to “allergenicity of genetically modified foods” in the title of the document, the present scope has been broadened to also include ingredients derived from fermentation processes using microorganisms that have not necessarily been genetically modified. Moreover, it includes “novel foods” in the broad sense, defined as foods that have not previously been on the a market regulated by a food authority such as the European Commission which is counselled by the European Food Safety Agency. Thus, whereas all genetically modified foods are necessarily novel foods, other food products may fall into this category such as a natural products or traditional foods from different regions of the world not hitherto marketed or consumed.

6.1 Case #1: ice-structuring protein derived from fish

Many fish species living in Arctic waters contain proteins in their blood that prevent ice crystal growth thus enabling survival at water temperatures below 0°C. This group of proteins is commonly referred to as antifreeze proteins, but more recently the term ice-structuring proteins (ISPs) has been proposed [56]. ISPs have a range of potential commer-
cial applications including those in food products and, for this reason, the protein has been cloned and expressed in the yeast *Saccharomyces cerevisiae* [57]. The protein investigated in this study was the so-called ISP Type III isoform HPLC 12 (hereafter referred to as ISP Type III) originating from the Arctic ocean pout (*Macrozoarces americanus*), in which it occurs at high concentrations (30 mg/mL) in the blood.

At the time of the study no data existed on allergy to ocean pout itself, although it was expected that fish-allergic individuals would react to ocean pout and also to eel pout, which lives in Scandinavian waters. It has later been confirmed that there is an extensive cross-reactivity between codfish, eel, eel pout, and ocean pout [58]. Before any food application could be considered it was essential to demonstrate that such cross-reactive individuals do not react to the novel protein even though they might react to ocean pout flesh containing Gad c1 analogues.

Initially, no homology could be found to any known allergen [59] at greater than five contiguous amino acids. After this a specific serum screening with IgE from fish-allergic individuals was performed [57]. Sera from 20 patients with a well-documented clinical history of fish allergy, positive in skin prick tests to ocean pout, eel pout and eel, were used. All of these demonstrated positive IgE-binding in vitro to extracts of the same fish. The sera also elicited histamine release in vitro in the presence of the same extracts. The ISP was negative in all cases in the same experiments [57]. Finally, it was demonstrated that during pepsin treatment the ISP were degraded to small fragments within less than 10 min [59]. Based on these results it was concluded that ISP is unlikely to cross-react with known fish allergens, and is not likely to act as a sensitizing protein.

### 6.2 Case #2: a five amino acid contiguous homology

Transglutaminase is an enzyme widely distributed in nature and can be found in diverse animal tissues, fish, and plants [60]. Natural microbial transglutaminase (m-TG) has been isolated from the organism *Streptocverticillum mobararse* [61] and is not a GMO product. m-TG is homologically different from transglutaminases found in plants and animals [62]. It has a molecular mass of 38 kDa and contains no saccharide or lipid moieties [63]. m-TG catalyzes the cross-binding of protein-bound glutamine and a primary amine such as lysine residues of proteins. The enzyme acts as a texturizing agent in prepared foods adding firmness, thermal stability and water-holding capacity when applied in seafood, meat products, noodles, pasta, dairy products, and baked goods.

For risk evaluation of m-TG it was not possible to find any cases of IgE sensitization toward a bacterial strain and, since the source of the product is considered nonallergenic, a specific serum screen was not possible. As described in an accompanying paper in this volume*, a sequence alignment in protein databases was made to compare the amino acid sequence of m-TG to all known allergens. The study demonstrated that, down to a match of six contiguous amino acids, there was no homology with any type of allergen, this meets the requirements of the decision tree. However, a match was found at the five contiguous amino acids level between m-TG and the allergen Gad c1. Due to the fact that Gad c1 is the major codfish allergen and extremely heat-stable [7, 64], a targeted serum screen of m-TG using sera from cod-allergic patients was performed. The potential cross-reactivity between m-TG and Gad c1 was investigated in both direct RAST and RAST inhibition of codfish using sera from 25 documented cod-allergic patients and an extract of raw codfish. No binding between patient IgE and m-TG was observed. Furthermore, the degradability in pepsin and trypsin were tested. Pepsin completely cleaved m-TG into small fragments within 1 min at pH 2.5 whereas trypsin failed to cleave all of the m-TG and a full-length band remained in the original position even after 48 h. Accordingly, it was concluded that no safety concerns with regard to the allergenic potential of m-TG in relation to fish products had been identified.

### 6.3 Case #3: a known food allergen

In order to study the influence of gene modifications of a less abundant protein on the expression of major allergens in a plant, a number cultivars of soybean were been tested [65]. Since it has been consistently difficult to identify soybean allergic individuals, the study relied on persons with a positive specific IgE response to soybean, but without a positive clinical reaction to soybeans. In the study eight cultivars of GMO soybeans (Round-up Ready®, Monsanto) were compared with ten cultivars of wild-type soybeans by means of RAST inhibition, SDS-PAGE followed by Western blotting, and histamine release from cord blood basophils that had been passively sensitized with sera with specific IgE to soybean. Finally, individuals with specific IgE to soybean were skin-tested with extracts of the soybeans. In the semiquantitative tests, such as RAST-inhibition and histamine release, the responses to extracts varied more than one order of magnitude, but no differences between the GMO and the wild-type soybeans could be detected. The pattern of allergens, * Pedersen, M.H., Hansen, T. K., Sten, E., Seguro, E. et al., Evaluation of the potential allergenicity of the enzyme microbial transglutaminase using the 2001 FAO/WHO Decision Tree. Mol. Nutr. Food Res. 2004, 48, DOI 10.1002/mnfr.200400014. This issue of MNF.
as determined by SDS-PAGE followed by Western blotting revealed the qualitative identity between the 18 cultivars. Finally, equivalent biological potencies were obtained in skin tests [65]. The variation observed within each of the two groups of the study emphasizes the importance of taking natural variation into consideration when comparing GMO and wild-type cultivars of the same plant. The large variation within the groups may be caused by factors such as varying genetic background (not related to the intended gene modification), different cultivation conditions, and variations in extractability and extract stability.

6.4 Case #4: a known allergen not hitherto recognized as a food allergen

To compensate for a low content of natural amylases, bioindustrially produced α-amylase is added to wheat flour to improve the leavening of the dough. One such α-amylase is derived from *Aspergillus oryzae* and is formulated as the product Fungamyl®, which has been used as an additive to flour for 40+ years. This preparation of α-amylase complies with the FAO/WHO JECFA and FCC recommended specifications for food-grade enzymes [66, 67] and is generally considered safe for human ingestion**. Occupational exposure to enzyme dust may, however, cause type I allergic sensitization and allergic symptoms like asthma and rhinitis, and urticaria may be elicited on subsequent exposure [68]. Preparations of α-amylase derived from *Aspergillus oryzae*, including Fungamyl®, have in several cases been reported to cause sensitization of workers in enzyme production plants [68], in pharmaceutical industries [69], or bakeries [70, 71]. Casuistic reports have appeared on food allergic reactions to α-amylase among occupationally sensitized patients [69–71]. In this study we aimed at using the methods of the left part of the ILSI decision tree to study the frequency of food allergy to the fungal α-amylase Fungamyl® in a population previously sensitized via the inhalation route. Moreover, a screening of 1000 persons from the general population was performed to estimate the frequency of IgE-sensitization to Fungamyl® (sensitization screening study) [72] and Vestergaard *et al.*, manuscript in preparation).

Eighteen patients with allergy to Fungamyl® were double-blind, placebo-controlled food challenged (DBPCFC) with 100 g of bread baked with a double amount of Fungamyl®. Bread baked without enzyme served as placebo. Fungamyl® coated onto the Pharmacia ImmunoCAP was used for the sensitization screening study [73]. The DBPCFC was divided into two phases: First one active and one placebo challenge was used. Eleven patients reacted to neither and left the study. Of the remaining seven persons, four reacted to active, and four to placebo (one patient reacting to both challenges), and all were submitted to three active and three placebo DBPCFC. Here, one patient reacted to one active challenge and five reactions to placebo were observed. All reactions were mild and the relatively large number of placebo reactors probably reflects a very low level of discrimination for symptoms. In the sensitization screening study none reacted positively with IgE to Fungamyl®. Thus, none of 18 patients with inhalation allergy to Fungamyl® expressed food allergy to bread baked with a double dose of the enzyme (95% c.i.: 0–14.5%). Since no sensitized persons were identified in the background population, we conclude that food allergic reactions to Fungamyl® are likely to be rare in the general population. Even in patients with inhalation allergy to the enzyme; inhalation allergy is not commonly accompanied by a corresponding food allergy.

6.5 Case #5: an antigen not hitherto known to bind IgE, but demonstrating cross-reactivity with known allergens

Several examples exist of allergic reactions caused by cross-reactive, plant-derived foods or by cross-reactivity between pollen and food allergens. In some studies, however, it cannot be absolutely ascertained whether birch pollen or plant-derived foods are the primary cause of sensitization since often a concomitant exposure to the food in question cannot be excluded. In order to study an example where there could be little doubt about the primary sensitization, we selected a new food that had never appeared on European market, *i.e.*, the so-called Nangai nut (*Canarium indicum*) natively grown on Polynesian Islands. We have evaluated the clinical and serological relevance of cross-reactivity between Nangai nut and pollen allergens [74].

Cross-reactivity was examined with direct RAST, RAST inhibition, and Western blot using sera from patients allergic to grass, birch, and mugwort pollen. None of the patients reported to have seen or eaten Nangai previously. Biological and clinical relevance of the cross-reactivity was investigated using histamine release test, skin prick test, and food challenge. Reactivity to Nangai was found in a subgroup of the pollen allergic patients. This cross-reactivity seems to be related, at least in part, to carbohydrate epitopes and had clinical consequences since a few patients tested with Nangai were positive upon challenge. The biological effects of Nangai on allergic patients were confirmed using histamine release and skin prick test [74].

Although Nangai is not a gene-modified food, rather a novel food, this example illustrates that IgE-binding epi-

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** Fungamyl is covered by a GRAS (Generally Recognized As Safe) petition filed with the FDA in the USA: GRASP 3G0016, Federal Register, Vol. 38, No. 112, June 1973.
topes may be present in foods and proteins to which subjects have never been exposed. This calls for a strategy to identify such epitopes. One such strategy would be allergenomics, a screening procedure where panels of sera from classes of allergic patients are used for detection of binding between IgE and “new” proteins or foods. It is likely that in some cases such a procedure will produce a number of false-positive reactions which will then have to be confirmed or ruled out by more biologically relevant techniques such as histamine release or skin testing. In this respect, the possibility of passive sensitization of cord blood basophils is interesting since this bypasses the need for the physical presence of donors.

7 Discussion and conclusion

In the ILSI Decision Tree from 1996, it was suggested that algorithms be developed to identify homology between a new protein and primary structures of known allergens [13] and this strategy has been sustained in the FAO/WHO guidelines [14]. Looking only at primary structure excludes the possibility of conformational epitopes, however, and new theoretical or experimental routes are required. The criticism raised toward the large number of protein sequences – including 43% of the proteins encoded by the human genome – clearly needs to be addressed in future revisions of the decision tree.

One interesting possibility which would add experimental evidence to the in silico methods for identifying novel food allergens may be the use of phage-display technology, where a high-throughput technology for identifying epitopes has been developed (U.S. Patent # WO 00/26230). In this system conserved epitope patterns are obtained by screening phage libraries that express random oligomeric 9- and 12-mers with human or murine antibodies against a variety of known allergens. The oligomers coding sequences that are expressed by immunoreactive phages, are subsequently subjected to DNA sequence analysis. Finally, alignment of the resulting sequences allows the identification of conserved epitope patterns, which are then collected in an epitope database. The database is continuously expanded with new epitope patterns. These patterns can be protein-specific, but most often are obtained from various proteins. Eventually, the antibody-type binding to a specific pattern is identified. Occasionally, patterns appear to be antibody-type specific. The 3-D structure of the protein is then screened with all the epitope patterns available in this epitope database. This screening is performed semi-automatically. The relevance of the identified sequences was assessed and confirmed by epitope engineering in identified IgG and IgE epitopes, and animal studies. Furthermore, it was shown that this approach allows prediction of cross-reactivity between proteins (Roggen, E. L., personal communication).

Serum-based testing may, in principle, comprise any detection of antigen-antibody binding between a food protein and an antibody of any given isotype. Moreover, the serum may be derived from human or animal sources. Even though an IgG immune response to a component in a food arguably classifies the component as an antigen, it is far from fully elucidated whether or how IgE-binding antigens, i.e., allergens, can be distinguished as a subgroup of all antigens.

In the hierarchy that exists among test systems for allergenicity, challenge of human patients is considered closest to the relevant biological response, i.e., elicitation of an actual allergic response, albeit under controlled and safe circumstances [8, 75]. The next level in the hierarchy is to use the skin as a restricted and localized area for challenge. This system obviously involves the skin mast cells which must be sensitized by IgE in order to respond to the offending allergen. Subsequent to the in vivo systems, the next step is to use the sensitized basophil granulocyte as a model for the sensitized mast cell present in the relevant organ of the patient. The serum-based tests represent yet a further step away from the patient as they do not give information on the functional relevance of the interaction between a potential allergen and IgE. A pure system can be obtained by immunochimical assays detecting IgE-allergen binding directly or indirectly by inhibition designs. By means of passive sensitization of basophils from a nonallergic donor such as cord blood or buffycoats from blood bank donors, it is possible to perform a practical biological test that may be used to identify the biological – albeit not the clinical – relevance of a protein cross-reacting with IgE from an actual patient.

The example from case #4, i.e., the screening of a background population of 1000 donors for allergy to Fungamyl®, illustrates that it is indeed possible to perform large screens without obtaining large numbers of false-positive sera. Admittedly, large logistic problems including establishment of relevant serum banks remain for practically implementing this strategy.

The ILSI Decision Tree presented in 1996 [13] has been further developed into the FAO/WHO guidelines [14]. It is evident that even if a protein has not previously been ingested, it may still cause IgE-binding and, in some cases, even elicit clinically relevant symptoms. Thus far, this possibility has been approached theoretically by algorithms identifying homology of short amino acid sequences between new proteins and already known allergens. The possibility of IgE cross-reactivity is then addressed by an experimental screening approach. By collection of large serum banks based on well-characterized patients, this test-
ing could be improved; this could be described as an allergenomic approach. New technologies are under way to refine the identification of IgE-binding epitopes, but we still do not have sufficient knowledge about the sensitization phase of food allergy. Thus, it cannot be excluded that a novel food or GMO will be developed, which has the same sensitizing potential as peanut, for example, since the present state of knowledge does not allow us to fully define what fully comprises an allergen.

8 References


[22] Spangfort, M. D., Mirza, O., Ipsen, H., van Neerven, R. J., et al., Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. J. Immunol. 2003, 171, 3084–3090.


