

# Prediction of Allergenicity of Gene-Modified Foods by Serum-Based Testing

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**ABSTRACT:** On the basis of applying the IFBC/ILSI decision tree in a number of cases, a refinement of the scheme is suggested. Large differences in allergenic potential may be obtained by altering the route of administration of an allergen. Because an inhalation allergen can induce symptoms at different threshold doses depending on whether it is introduced via the inhalation or oral route, we propose that double-blind, placebo-controlled food challenges be performed in all cases of inhalation allergens being present in foods. Even proteins never previously ingested may cause IgE binding and elicit clinically relevant symptoms.

**KEYWORDS:** serum-based testing; animal models; allergenicity; basophils; antibody binding; double-blind, placebo-controlled food challenges inhalation allergen; risk assessment

## INTRODUCTION

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. Animal models are thoroughly discussed in other parts of this volume, and even though serological testing is one possible readout parameter of animal models, it should be discussed in conjunction with other aspects of setting up a given model. It should be mentioned, however, that the methods employed for detection of antibody binding to an antigen are essentially identical in animal and human systems.

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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, that is, allergens, can be distinguished as a subgroup of all antigens. Accordingly, measurements of human IgG or other non-IgE isotype responses to food antigens will not be discussed further. Thus in this paper, serum-based testing is considered to encompass testing of binding between human IgE and components in foods. This binding may be determined directly or indirectly, and the results may be expressed quantitatively or qualitatively.

In the hierarchy that exists among test systems for allergenicity, challenge of human patients is considered closest to the relevant biological response, that is, elicitation of an actual allergic response, albeit under controlled and safe circumstances.<sup>1,2</sup> 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. Leaving 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 a further moving 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 immunochemical assays detecting IgE–allergen binding directly or indirectly by inhibition designs. Moreover, basophils from a nonallergic donor, such as from cord blood, may be used as a reagent; they are then sensitized by IgE derived from an actual patient. Technological aspects and the application of the tests are reviewed by Poulsen<sup>2</sup> and Taylor and Lehrer.<sup>3</sup>

Whether the readout is a functional parameter, such as symptoms after challenge or histamine release or IgE binding, as in serum-based tests, it must be emphasized that the relation between antibody binding potentials and antibody production potentials is not known.

## METHODS

### *IgE Reactivity to Allergen Extracts (RAST-type Methods)*

Since IgE was first purified and anti-IgE antisera became available,<sup>4–7</sup> immunoassays have been designed for specific IgEs using either radiolabeled allergen<sup>8,9</sup> or radiolabeled anti-IgE antibody, as in the RadioAllergoSorbent Test (RAST).<sup>10</sup> The initial design of the RAST was based on the use of dextran-derived materials,<sup>8,10</sup> but later solid phases have comprised the widely used paper discs,<sup>11</sup> aluminium hydroxide gel,<sup>12</sup> polystyrene tubes,<sup>13</sup> cellulose polymers,<sup>14,15</sup> and magnetic microparticles.<sup>16</sup> Besides varying the solid

phase, the detection principle has also been modified to comprise enzyme-catalyzed reactions (enzyme-linked allergosorbent assays, EAST), fluorescence, and chemiluminometric procedures. Reviews of the available technologies and a discussion of method evaluation have been given in Matsson *et al.*<sup>17</sup> and Bindslev-Jensen and Poulsen.<sup>18</sup>

It is possible to use RAST-type methods either directly or indirectly, that is, as inhibition RAST, to detect IgE binding to an allergen. In the direct method the extract is coated to the solid phase, which makes it necessary to check that the relevant component is 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, that is, the direct RAST to be inhibited, represents the relevant binding between the component and specific IgE.

### *Immunoblotting and Comparable Methods*

A qualitative description of the IgE-binding antigens in a mixture may be obtained by subjecting the allergen extract to a primary separation. Such analyses are based on molecular weight (SDS-PAGE), isoelectric point (isoelectric focusing), or both (2-D gels); or they are based on combinations of physico-chemical and immunochemical characteristics (crossed immunoelectrophoresis, CIE) as separation principles. After a form of fixation of the separated pattern of components, the IgE-binding antigens may be detected by a secondary immunodetection involving incubation with allergy serum followed by a labeled anti-IgE antibody. Examples of such methods are Western blotting of nitrocellulose membranes obtained from SDS-PAGE molecular weight separations or the crossed radioimmuno-electrophoresis (CRIE) method, where an IgE-immunoradiometric assay is performed on the top of the pattern of precipitates in a CIE gel. A comparison of different methods has suggested that the ideal result is only obtained by a combination of the different methods.<sup>19</sup>

### *Passive Sensitization of Human Basophils*

The principle of the method is to challenge sensitized basophils with allergen that 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 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 on basophils with no specific IgE to ensure that no nonspecific histamine release, that is, caused by cytotoxicity, takes place. Histamine can be measured fluorometrically after coupling to a fluorophore (*o*-phthaldialdehyde). Alternative

methods for detection of histamine are immunochemical: the Immunotech Radioimmunoassay<sup>20</sup> and the automated fluorometric histamine assay.<sup>21</sup> Agreement between the three methods has been established.<sup>22,23</sup>

In one application of the histamine release method, glass fiber-coated microtiter plates are used for separation of histamine from other constituents of the assay.<sup>24,25</sup> Heparinized, washed blood may be used without further separation, but the use of whole blood or gradient-enriched basophil suspensions has also been described. Blood (25  $\mu$ l) is stimulated in glass fiber-coated microtiter plates with dilutions of allergen extracts (15:1) for 1 hr at 37°C. Three 10-fold dilutions of anti-IgE are used as the positive control, and medium (PIPES buffer + magnesium and calcium) are used as negative controls. All stimulations are performed *in duplo*, and each stimulation produces one histamine determination. Histamine is subsequently separated from the cellular suspension since it is absorbed onto the glass fibers of the microtiter plate. The plates are washed extensively, and the histamine can be released by a change in the pH and finally measured fluorometrically after coupling to a fluorophore (*o*-phthalaldehyde). The histamine determination is calibrated by histamine standards in the medium, which are incubated in glass-fiber wells in parallel with the blood. A number of biogenic amines (spermidine, cadaverine), histamine metabolites, and histidine have been tested and found not to interfere with the assay.<sup>25</sup> Because some allergen extracts have been reported to contain histamine,<sup>26</sup> it is essential that the histamine content of each allergen extract is checked when a new batch of allergen extract is used in the analysis. 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.

## USE OF SERUM-BASED TESTING FOR RISK ASSESSMENT

From the above-mentioned focus on human IgE-based testing, it follows that the scope for these tests will be antigens for which a previous IgE immune response has occurred. As will be discussed in greater detail below, this does not, however, completely limit the antigens to those already described in the literature as allergens. Four scenarios are described, three of which have been investigated with serum-based methods.

### *Scenario 1: 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 soy have been tested.<sup>27</sup> Because it has been consistently difficult to identify soybean-allergic individuals, the study relied on persons with a positive, spe-

cific IgE response to soybean, but without a positive clinical reaction to soybeans. In the study eight cultivars of GMO soybeans (Round-up Ready<sup>®</sup>, Monsanto) were compared with 10 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 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, 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.<sup>27,28</sup> 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.

### ***Scenario 2: A Known Allergen Not Hitherto Recognized as a Food Allergen***

To compensate for a low content of natural amylases, bioindustrially produced  $\alpha$ -amylase is added to wheat flour to improve the leavening of the dough. One such  $\alpha$ -amylase is derived from *Aspergillus oryzae* and is formulated as the product Fungamyl<sup>®</sup>, which has been used as an additive to flour for 40+ years. This preparation of  $\alpha$ -amylase complies with the FAO/WHO JECFA- and FCC-recommended specifications for food-grade enzymes<sup>29,30</sup> 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, rhinitis, and urticaria may be elicited upon subsequent exposure.<sup>32</sup> Preparations of  $\alpha$ -amylase derived from *Aspergillus oryzae*, including Fungamyl, have in several cases been reported to cause sensitization of workers in enzyme production plants,<sup>32</sup> in pharmaceutical industries,<sup>33</sup> or bakeries.<sup>34,35</sup> Reports have appeared on food allergic reactions to  $\alpha$ -amylase among occupationally sensitized patients.<sup>31–33</sup> In this study we aimed at using the methods of the left side of the ILSI decision tree (FIG. 1) for studying the frequency of food allergy to the fungal  $\alpha$ -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.<sup>36,37</sup>

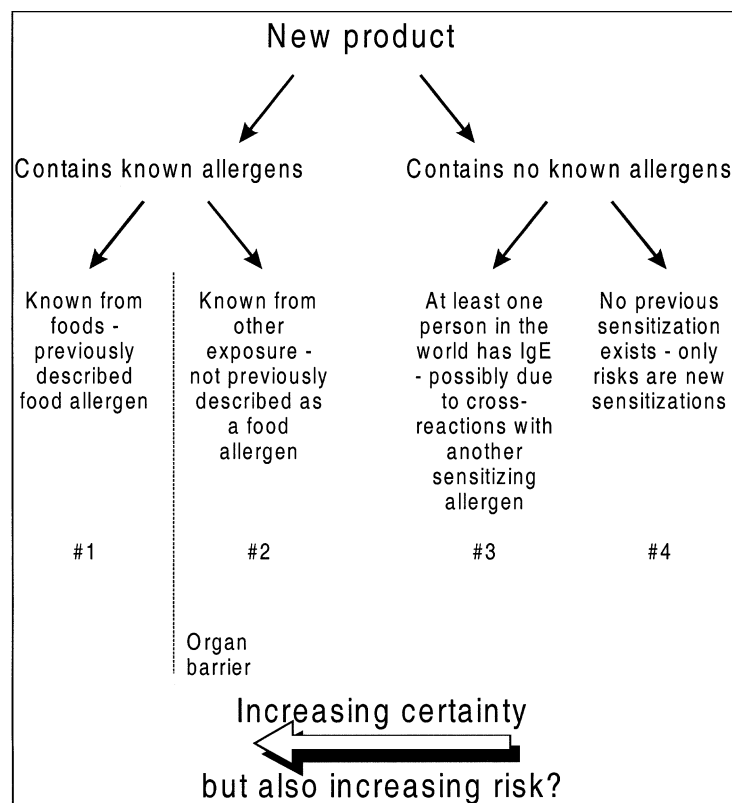


**FIGURE 1.** A refined decision tree.

Eighteen patients with allergy to Fungamyl were double-blind, placebo-controlled food challenged (DBPCFC) with 100 g of bread baked containing 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.<sup>38</sup>

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 plus three placebo DBPCFCs. In this case, 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%). Because 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 among patients with inhalation allergy to the enzyme, it is not commonly accompanied by a corresponding food allergy.

***Scenario 3: 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 other 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 in which there could be little doubt on the primary sensitization, we selected a new food that had never appeared on the European market, the so-called Nangai nut (*Canarium indicum*), natively grown on the Polynesian islands. We have evaluated the clinical and serological relevance of cross-reactivity between Nangai nut and pollen allergens.<sup>39</sup>

The 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 having seen or eaten Nangai previously. The biological and clinical relevance of the cross-reactivity was investigated using the histamine release test, the skin prick test, and food challenge.

Reactivity against 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 the histamine release and skin prick tests.<sup>39</sup>

Although Nangai is a novel food, not a gene-modified food, this example illustrates that IgE-binding epitopes may be present in foods and proteins to which humans have never been exposed. This calls for a strategy to identify such epitopes. One such strategy—*allergenomics*—would be a screening procedure in which 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 such a procedure will, in some cases, 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, because this bypasses the need for the physical presence of serum donors.

On the other hand, the example from scenario 2, that is, the screening of a background population of 1000 donors against Fungamyl, illustrates that it is indeed possible to perform large screens without obtaining large numbers of false-positive sera. Admittedly, large logistical problems including establishment of relevant serum banks remain for using this strategy.

In the 1996 ILSI decision tree, it was suggested that algorithms be performed to identify homology between a new protein and primary structures of known allergens.<sup>40</sup> Looking only at the primary structure excludes the possibility of conformational epitopes, however, and new theoretical or experimental routes are required. One such interesting possibility may be the use of phage-display technology, for which 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, expressing random oligo-9 and 12-mers, with human or murine antibodies against a variety of known allergens. The oligomers' coding sequences, which are expressed by immunoreactive phages, are subjected to DNA sequence analysis. Finally, alignment of the resulting sequences allows the identification of conserved epitope patterns that are 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 different 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 semiautomatically. The relevance of the identified sequences was assessed and confirmed by epitope engineering in identified IgG and IgE epitopes and in animal studies. Furthermore, it was shown that this approach allows us to predict cross-reactivity between proteins (Erwin L. Roggen, personal communication).

#### ***Scenario 4: A Substance That Cannot Be Associated with Any Human IgE-Binding Activity***

Because the fourth scenario is defined by the absence of any human IgE binding, serum-based testing is not relevant in this context. The risk of this subgroup is exclusively related to the new sensitizing properties of a given protein or food. Animal models are discussed elsewhere in this volume, and theoretically these may in time be supplemented with *in vitro* immunization systems or systems in which matrix or adjuvant effects may be investigated.<sup>41</sup>

## CONCLUSION

In the IFBC/ILSI decision tree presented in 1996,<sup>40</sup> two main routes were identified: Either the protein in question is already known as an allergen, or it is unknown in that respect. In the former case, standard IgE tests followed by skin testing and clinically controlled food challenges were suggested as the route to assessing the potential for inducing allergic reactions, whereas in the latter case, other methods, such as algorithms for detecting linear allergenic epitopes or digestability studies, were suggested. We have attempted to use the IFBC/ILSI decision tree in a number of cases, and on the basis of these we can now suggest a refinement of the scheme. As illustrated above, there may be a large difference in allergenic potential, depending on whether an inhalation allergen is administered via the inhalation or via the oral route. We have conceptualized this difference with the term *organ barrier*, which illustrates the situation in which modifications in administration route result in different abilities or different threshold dosages for induction of symptoms. Accordingly, we propose double-blind, placebo-controlled food challenges to be performed in all cases of inhalation allergens present in foods, because specific IgE may not in itself be a valuable indicator of allergenic potential.

Also evident from the study mentioned above is the fact 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. So far, this possibility has only been approached theoretically by algorithms identifying homology of strings of amino acids between new proteins and already known allergens. As suggested above, it may be possible to address the possibility of IgE cross-reactivity by an experimental screening approach described as *allergenomics*. Moreover, new technologies are under way to refine the identification of IgE-binding epitopes.

In relation to the risk assessment, it is important to separate the concept (lack of) knowledge from that of (lack of) risk. As indicated in FIGURE 1, it may be possible to establish with a high degree of certainty that a new protein causes clinically relevant food allergy in one individual, that is, by double-blind, placebo-controlled food challenges. If the symptoms are not severe, however, and the prevalence of patients with the relevant allergy is low, the total risk of the protein may be limited. On the other hand, the lack of a previous history of sensitization may not be relevant if the protein has not previously been ingested in the new context. On theoretical grounds, it may still be possible to create a food with the same sensitizing potential as, for example, peanut, since the present state of knowledge does not allow us to fully define what makes an allergen an allergen.

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