

In-vitro diagnosis: serum-based methods used for risk assessment of allergenic food

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This review on in-vitro diagnostic methods focuses on the use of methods to perform risk assessment on foods. Based on the International Life Science Institute (ILSI) risk decision tree, the methods are discussed and three scenarios are suggested:

(i) testing for a well-known allergen; (ii) testing for a well-known allergen, but with no previous history of food allergy; and (iii) testing for unknown allergens and cross-reactivity with known allergens. *Curr Opin Allergy Clin Immunol* 1:249–254. © 2001

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Abbreviations

DBPCFC	double-blind, placebo-controlled food challenge
GMO	genetically modified
RAST	radioallergosorbent test
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis

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Introduction

In-vitro diagnosis of food allergy consists of serological and cellular tests. The former includes radioallergosorbent test (RAST)-type methods for specific IgE, as well as more research-based methods such as immunoblotting in which IgE reactivity towards individual components in an extract is determined. Cellular methods consist of mediator release (histamine, leukotrienes) from effector cells, and the determination of specific T and B cells.

Serum-based testing may in principle consist of 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, but animal models will not be discussed in this article. However, the methods employed for the detection of antibody binding to an antigen are essentially identical in animal and human systems.

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, and the use of IgG methods in food allergy remains a controversial issue [1,2•]. Accordingly, measurements of human IgG or other non-IgE isotype responses to food antigens will not be discussed further. In the present context, serological testing is considered to encompass the 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, the challenge of human patients is considered to be closest to the relevant biological response, i.e. the elicitation of an actual allergic response, albeit under controlled and safe circumstances [3,4]. 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. Serum-based tests represent a further move away from the patient, because 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 non-allergic donor such as cord blood may be used as a reagent, which is then sensitized by IgE derived from an actual patient. The technological aspects and applications of the tests are reviewed in Poulsen [4] and Taylor and Lehrer [5].

In-vitro tests may be performed for a number of reasons. As discussed above, the tests may rarely produce a diagnosis, but provided they have a reasonable specificity, they may be used for the exclusion of foods from further testing. Moreover, in-vitro tests may provide an indication of the level of security that should be exercised in subsequent challenge tests. The present review will focus on the use of in-vitro diagnostic tests for determining the allergenic potential of a food. This viewpoint is relatively new, but deserves exploration because it suggests possibilities to learn more about allergenic foods, and in turn provides us with tools for the prevention of exposure and allergic reactions.

Methods

Basically, the in-vitro methods for detection of sensitization may be divided into RAST type techniques, where reactivity towards an extract is determined; immunoassays based on a previous separation of the extract providing knowledge of sensitization to individual allergens; and finally biological assays where also the quality of IgE-crosslinking contributes to the response.

IgE reactivity to allergen extracts (radioallergosorbent test-type methods)

Since IgE was first purified and anti-IgE antisera became available [6–9] immunoassays have been designed for specific IgE using either radiolabelled allergen [10,11] or radiolabelled anti-IgE antibody as in the RAST [12]. The initial design of the RAST was based on the use of dextran-derived materials [10,12], but later solid phases have comprised the widely used paper discs [13], aluminium hydroxide gel [14], polystyrene tubes [15], cellulose polymers [16,17], and magnetic microparticles [18]. Besides varying the solid phase, the detection principle has also been modified to consist of enzyme catalysed reactions (enzyme-linked allergosorbent assays), fluorescence, and chemiluminometric procedures. Reviews of the available technologies and a discussion of method evaluation have been given in Matsson *et al.* [19] and Bindeslev-Jensen and Poulsen [20].

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 onto 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, i.e. the direct RAST that is 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 (sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SDS–PAGE), isoelectric point (isoelectric focusing) or both (two-dimensional gels), or combinations of physicochemical and immunochemical characteristics (crossed immunoelectrophoresis) 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 labelled anti-IgE antibody. Examples of such methods are Western blotting of nitrocellulose membranes obtained from SDS–PAGE molecular weight separations or the crossed radioimmunoelectrophoresis method, in which an IgE immunoradiometric assay is performed on the top of the pattern of precipitates in a crossed immunoelectrophoresis gel. A comparison of different methods has suggested that the ideal result is only obtained by a combination of different methods [21].

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 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 non-specific histamine release, i.e. caused by cytotoxicity, takes place. Histamine can be measured fluorometrically after coupling to a fluorophore (o-phthaldialdehyde). Alternative methods for the detection of histamine are immunochemical: the immunotech radioimmunoassay [22] and the automated fluorometric histamine assay [23]. Agreement between the three methods has been established [24,25].

In one application of the histamine release method glass-fibre coated microtitre plates are used to separate histamine from other constituents of the assay [26,27]. Heparinized washed blood may be used without further separation, but the use of whole blood or gradient-enriched basophil suspensions has also been described. Twenty-five μ l blood are stimulated in glass-fibre coated

microtitre plates with dilutions of allergen extracts for 1 h at 37°C. Three tenfold dilutions of anti-IgE are used as positive controls, and medium (PIPES buffer plus magnesium and calcium) is used as negative controls. All stimulations are performed in duplicate, and each stimulation produces one histamine determination. Histamine is subsequently separated from the cellular suspension because it is absorbed by the glass fibres of the microtitre 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-fibre 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 [27]. As some allergen extracts have been reported to contain histamine [28], 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, because new and unproved preparations of allergens or other offending substances may have cytotoxic activities, a control should always be carried out using a non-allergic 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 further, this does not, however, completely limit antigens to those already described in the literature as allergens.

Four scenarios are now described, three of which have been investigated with serum-based methods.

Scenario 1: a known food allergen

An area that has recently attracted attention is fish gelatine, which has found increasing use. It has been investigated whether individuals with a previous IgE-mediated allergy to fish have any risk of allergic reaction upon the ingestion of fish gelatine. By means of enzyme-linked immunosorbent assay and immunoblotting it was demonstrated that individuals with preformed IgE to fish would in some cases also have a response to fish gelatine [29]. That study did not investigate whether there were any clinical consequences of the IgE response.

Another food was studied in order to learn about 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 as an example of this [30]. As it has been consistently difficult to identify soybean allergic individuals, the study relied

on individuals with a positive specific IgE response to soybean, but without a positive clinical reaction to soybeans. In the study, eight cultivars of genetically modified organisms (GMO) soybeans (Round-up Ready, Monsanto St Louis, MO) 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 [30] (E. Sten, P. Stahl Skov, S. Bode-Andersen, A.M. Torp, A. Olesen, U. Bindslev-Jensen, L.K. Poulsen, C. Bindslev-Jensen, 2001, in preparation). 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 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 over 40 years. This preparation of α -amylase complies with the FAO/WHO JECFA (JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES) and FCC (Food Chemicals Codex) recommended specifications for food grade enzymes [31,32], and is generally considered to be safe for human ingestion. (Fungamyl is covered by a Generally Recognized As Safe (GRAS) petition filed with the Food and Drug Administration in the USA: GRASP 3G0016, Federal Register, Vol. 38, no. 112, June 1973.) Occupational exposure to enzyme dust may, however, cause type I allergic sensitization, and allergic symptoms such as asthma, rhinitis, and urticaria may be elicited on subsequent exposure [33]. Preparations of α -amylase derived from *A. oryzae*, including Fungamyl have in several cases been reported to cause sensitization of workers in enzyme production plants [33], in pharmaceutical industries [34], or bakeries [35,36].

Casuistic reports have appeared on food allergic reactions to α -amylase among occupationally sensitized patients [34–36]. In the study by Skamstrup Hansen *et al.* [37] we aimed at using the methods of the left part of the ILSI decision tree for studying 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 individuals from the general population was performed to estimate the frequency of IgE sensitization to Fungamyl (sensitization screening study) [37] (H. Vestergaard, K. Skamstrup Hansen, L. Nüchel Petersen, C. Bindselev-Jensen, L.K. Poulsen, 2001, in preparation).

Eighteen patients with allergy to Fungamyl were tested with double-blind, placebo-controlled food challenge (DBPCFC) with 100 g 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 [38].

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 individuals, four reacted to active, and four to placebo (one patient reacting to both challenges), and all were submitted to three active plus 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.

Therefore none out of 18 patients with inhalation allergy to Fungamyl expressed food allergy to bread baked with a double dose of the enzyme (95% confidence interval 0–14.5%). Because no sensitized individuals were identified in the background population, we concluded that food allergic reactions to Fungamyl were likely to be rare in the general population. Even in patients with inhalation allergy to the enzyme, this 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, because a concomitant exposure to the food in question often 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, i.e. the so-called nangai nut (*Canarium indicum*) natively grown on Polynesian Islands. We evaluated the clinical and serological relevance of cross-reactivity between the nangai nut and pollen allergens [39].

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, skin-prick test and food challenge.

Reactivity against nangai was found in a subgroup of pollen-allergic patients. This cross-reactivity seems to be related, at least partly, to carbohydrate epitopes and may have clinical consequences because a few patients tested with nangai were positive upon an unblinded challenge. The biological effects of nangai on allergic patients were confirmed using histamine release and skin-prick test [39].

Although nangai is not a gene-modified food but a novel food, this example illustrates that IgE-binding epitopes may be present in foods and proteins to which man has 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 the detection of binding between IgE and ‘new’ proteins or foods. It is likely that such a procedure in some cases 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, because this bypasses the need for the physical presence of serum donors.

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

In the ILSI decision tree from 1996 it was suggested to perform algorithms to identify homology between a new protein and primary structures of known allergens [40]. 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, in which a high throughput technol-

ogy for identifying epitopes has been developed (US patent no. 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 oligo-mers 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, which 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 three-dimensional 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 the prediction of cross-reactivity between proteins (Dr E.L. Roggen, personal communication).

Scenario 4: a substance which cannot be associated with any human IgE binding activity

As the fourth scenario is defined by the absence of any human IgE binding, serum-based testing is not relevant in this context.

Conclusion

In the ILSI decision tree presented in 1996 [40] 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 digestibility studies were suggested. We and others have attempted to use the ILSI decision tree in a number of cases, and based on 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 by the term 'organ barrier', which illustrates the situation in which modifications in administration route result in the different ability or a different threshold dosage for the induction of symptoms. Accordingly, we propose DBPCFC to be made in all cases of inhalation allergens present in foods,

because specific IgE may not in itself be a valuable indicator of an allergenic potential.

Also evident from the above-mentioned study 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 the 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 refining the identification of IgE-binding epitopes.

In relation to risk assessment, it is important to separate the concept (lack of) knowledge from that of (lack of) risk. It is possible to establish with a high degree of certainty that a new protein causes clinically relevant food allergy in one individual, i.e. by DBPCFC. However, if the symptoms are not severe, 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 e.g. peanut, because the present state of knowledge does not allow us to define fully what makes an allergen an allergen.

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