Review

Allergen-specific IgE testing in the diagnosis of food allergy and the event of a positive match in the bioinformatics search

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Current documents on risk assessment of genetically modified foods recommend including IgE-binding tests on sera from allergic patients. However, there is no generally accepted recommendation on technical aspects of the testing procedures or on the interpretation of the results, despite that fact that both false positive and false-negative results may be caused by variability of the test procedures. The present article discusses the state-of-the-art of serological test procedures for qualitative and quantitative determination of specific IgE and interpretation of test results. It is emphasized that the use of sera from clinically well-characterized subjects is of high importance. In the case of a positive test result, the biological activity of the detected IgE antibodies, *i.e.*, the potential to trigger mediator release from basophils or mast cells in an allergen-specific manner, should be taken into account. However, present data also indicate that validation of such mediator release tests is required, both in terms of experimental protocols and with respect to correlation of the test results with the clinical situation. Further studies are also required to prove the usefulness of targeted serum screening, *i.e.*, the testing of gene products from organisms not known to be allergenic with sera from subjects allergic to related species.

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1 Introduction

The gold standard for diagnosis of food allergy is the double-blind placebo-controlled food challenge (DBPCFC) [1, 2]. Its laborious nature and safety risks make it less suitable as a routine diagnostic tool [3]. Therefore, its use is largely restricted to research settings in academic hospitals. The results of DBPCFC are essential to establish the relation between other diagnostic approaches and the clinical situation and thus, DBPCFC also represents the only definitive proof for allergenicity of a given novel food. However, in clinical practice, skin prick tests (SPT), and *in vitro* IgE measurements such as RadioAllergoSorbent Test (RAST), ELISAs, or fluorescence enzyme immunoassays (CAP™-FEIA) are generally used. These tests are high-throughput tools for demonstrating sensitization, but not allergy, to foods.

To assess the potential allergenicity of novel foods and proteins, DBPCFC is not a very practical approach and consequently, procedures for screening such products with sera from allergic subjects were developed. For example, the FAO/WHO decision tree (Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/y0820e/y0820e00.htm) developed in 2001 for evaluation of the potential allergenicity of genetically modified foods suggests detailed procedures for serum screening with relevant source organisms, as follows:

"Specific serum screening for the expressed protein […]" focuses "on assessment of the possible allergenicity of the expressed protein using sera from patients allergic to the

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Abbreviations: DBPCFC, double-blind placebo-controlled food challenge; **HR,** histamine release; **RAST,** RadioAllergoSorbent Test; **SPT,** skin prick test

source material […].” If a positive response is found, the expressed protein should be considered allergenic. If no such sera exist or none are found positive, crossreactivity 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 […]. For this “targeted serum screen”, 6 groups of source organisms are distinguished: yeast/molds, monocots, 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.”

The content of the WHO/FAO Consultation document is discussed in detail elsewhere [4]. The assessment strategy was developed further by the Codex Alimentarius Commission and implemented into a Codex Guideline (Codex Alimentarius Commission, 2003, Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents). One modification that was introduced by the Codex experts was that targeted serum screening was currently considered to be of limited value and only recommended as a future tool because sufficient validation data were lacking. However, targeted serum screens have been applied in the allergy assessment of non-genetically modified organism (GMO) novel foods or novel proteins aimed to be used as food ingredients [4]. It should be emphasized that targeted serum screening is aimed at detecting crossreactivity between a novel protein and an existing allergen which has induced an IgE-response in one or more individuals. Thus the scope of this step in the risk evaluation is not to evaluate sensitizing properties of the novel protein, but to evaluate potential allergy-eliciting properties in previously sensitized allergic patients.

This chapter focuses on the utility and requirements for validation of different methods for measuring specific IgE in the diagnosis of food allergy and for assessing the allergenicity of novel proteins and novel foods.

2 General considerations for diagnostic tests

If a positive challenge test result is accepted as the reference for the clinical situation, sensitivity of any diagnostic test is the percentage of positive tests in a population with confirmed food allergy, whereas specificity depends on the proportion of false positive results in a control population without the respective food allergy. In food allergy diagnosis, sensitivity may be affected by low allergen quality, or by low abundance of an important allergen in the food extract, whereas crossreactivity is the main factor leading to decreased specificity of diagnostic tests. It is important to note that specificity in regard to the clinical situation is different from specificity of a serological assay for antibody quantification, and that this “clinical specificity” of any *in vitro* test systems will strongly depend on the selection of control groups. This means that in many cases it is not the *in vitro* assay, but the immune system of the patients which causes, for example, a false positive result: IgE antibodies against the allergen are present and correctly detected, but do not cause symptoms of food allergy. Provided that high quality allergens are used (*i.e.*, relevant allergen molecules not degraded and present in appropriate concentrations), the sensitivity of the tests discussed below is usually high, resulting in a high negative predictive value. This means that, if biologically active IgE cannot be detected, it is very unlikely that a patient will be allergic to a given food or food protein. Examples for reduced sensitivity caused by allergen degradation are foods containing homologs of the major birch pollen allergen, Bet v 1 [5–7]. Low sensitivity due to low abundance of major allergens was reported for, *e.g.*, hazelnut [8, 9] and soybean [10]. Low clinical specificity caused by crossreactivity was again found for birch pollen-related foods [7, 11, 12], for determination of specific IgE to foods (especially cereals and legumes) in grass pollen allergic patients [13, 14], for legumes [15, 16] and fish [17, 18].

Over the past years, it has become clear that IgE antibodies against some allergenic structures show little correlation to the expression of clinical symptoms (*e.g.*, crossreactive carbohydrate determinants) [19–23]. Others tend to cause selectively mild symptoms. This is the case for Bet v 1-related allergens in several foods such as apple and hazelnut; although, for some foods containing allergens with lower sequence homology to Bet v 1 such as carrot [6], celery [24], and soybean [10, 25], a higher percentage of systemic reactions has been reported. Finally, some allergen groups such as lipid transfer proteins (LTPs) and 2S-albumins are risk factors for severe systemic reactions [26–31]. Knowledge on the sensitization profile of allergic patients against individual allergens may thus be used to refine clinical diagnosis and prognosis, and adapt strategies for prevention. Moreover, it has implications for the selection of sera to be used in allergy assessment procedures.

3 Methods

3.1 SPT

In allergology, SPT is widely used as a diagnostic tool [32, 33]. The test measures biological activity of specific IgE

antibodies (*i. e.*, activation of mast cells in the skin) and can be evaluated semiquantitatively, but does not provide information concerning absolute quantities of specific IgE. For many practitioners it is nevertheless the assay of choice for the diagnosis of allergy, because results are obtained rapidly (within 15 min) and are easy to understand for the patient, as the reactivity is actually experienced. SPT for the diagnosis of food allergy is frequently performed as prick-to-prick test with fresh foods, because commercially available food extracts often result in poor sensitivity due to lability of important allergens during extraction and storage. As mentioned, the best characterized examples of such labile allergens are Bet v 1 homologs in fruits like Mal d 1 in apple [34, 35], Pru p 1 in peach [26], or Pru av 1 [36] in cherry. Disruption of fruit tissue triggers oxidative processes by polyphenol oxidases and peroxidases, resulting in almost complete loss of IgE-binding potency [5, 37]. Already in the early 1980s, Björksten *et al.* [38] described an extraction method that prevents such loss of allergenicity, but some of the reagents used are not compatible with *in vivo* application. Later, a low-temperature, acetone powder method was introduced and successfully used to produce extracts that could safely be applied in SPT [39, 40]. For most of the commercially available fruit extracts such precautions are unfortunately not taken, resulting often in poor sensitivity. Furthermore, most of these products are poorly standardized compared to extracts of inhalant allergen sources like grass pollen or house dust mites. Extreme inter- and intracompany differences in extract composition have been demonstrated for, *e. g.*, hazelnut SPT reagents [41]. Taken together these shortcomings of commercial food SPT extracts have resulted in widespread use of the prick-to-prick method with fresh foods as diagnostic tool [42]. Of course, standardization of this approach is virtually impossible, but at least the active allergenic ingredients are present.

An alternative that has more recently been used on a limited scale is SPT with purified (natural or recombinant) allergens [43]. Such reagents have the advantage that they can more easily be standardized and that they are potentially more stable. As mentioned, an additional advantage of diagnostic tests with purified allergens, so-called component-resolved diagnosis, is that the predictive value for the clinical presentation is improved [44].

SPT can of course also be used to assess possible allergenicity of novel foods. Drawbacks of this approach compared to *in vitro* antibody assays are ethical concerns and the lower overall reproducibility of the method. A major benefit is that not only IgE binding, but also the biological activity of allergens is measured. Since allergen quality is the major factor that affects the sensitivity of SPT it has to be emphasized that problems of allergen extraction described here also apply to *in vitro* test allergens.

3.2 *In vitro* assays for quantitative determination of specific IgE

The introduction of the RAST marked the beginning of *in vitro* IgE testing [45 – 48]. The initial design of the RAST was based on the use of dextran-derived materials [47] but later other solid phases were developed including the widely used paper disks [48], aluminum hydroxide gel [49], polystyrene tubes [50], cellulose polymers [51, 52], and magnetic microparticles [53]. Besides varying the solid-phase, the detection principle, comprising enzyme catalyzed reactions (enzyme-linked allergosorbent assays, EAST), fluorescence, and chemiluminometric procedures has also been modified. Reviews of the available technologies and a discussion of method evaluation have been given [54, 55]. In a newer version of the labeled allergen technique, all IgE is captured by a paramagnetic solid-phase with anti-IgE, and biotinylated allergen is used for detection of allergen-specific IgE [56].

Many of these techniques have been commercialized. Technologies like ImmunoCAP by Pharmacia Diagnostics, AlaSTAT by Diagnostic Products Company (DPC) and ADVIA Centaur by Bayer Diagnostics are available for automated high-throughput diagnostic testing. The former assay uses immobilized allergens; the latter two are based on liquid allergens. Most of the commercial test systems include purified IgE as a reference curve parallel to specific IgE-determination and thus allow a reliable quantification of allergen-specific IgE with sufficient sensitivity, and cut-off levels for positivity are provided as (arbitrary – since there is no absolute traceability to an international recognized standard) International Unit (IU) *per* milliliters of IgE (0.35 IU/mL in most of the cases). However, method-specific differences exist [52 – 55]. In contrast to commercial systems, many in-house tests for specific serum IgE used by researchers lack a validated reference system and use ill-defined cut-off levels for positivity. Unspecific binding of anti-IgE reagents used in commercial test systems is usually excluded during assay validation by the manufacturer, and has not been reported in the literature. However, the possibility of unspecific binding has to be carefully addressed during development of in-house ELISA systems.

Food extracts for application in *in vitro* systems can be made taking all precautions needed to preserve their IgE-binding capacity, without being limited by restrictions for *in vivo* use. As mentioned, false-negative test results are, generally speaking, not an issue for *in vitro* food allergy diagnostic tests with proven performance, provided that the presence of the relevant allergen molecules at sufficient levels in the test extract can be ensured.

Available *in vitro* tests for specific IgE are often setup with saturating allergen concentrations. Under these conditions, it can be expected that low-affinity IgE antibodies are also

detected. These conditions may contribute to frequently observed low specificity of tests for food allergy. To predict with 95% certainty whether a positive result translates into clinical allergy, several groups have proposed to determine "clinically relevant" decision points for specific IgE levels [57, 58]. Unfortunately, decision points for the same food differed significantly between the reports [57–60]. Even if there was close agreement between observed IgE thresholds, individual patients will exist who have IgE titers below the threshold but have clinical allergy. From a safety point of view, absolute use of such thresholds is therefore not advisable. Assays with a better clinical prognostic value will have to take the biological activity of specific IgE antibodies into account, *i.e.*, avidity of the interaction between allergen and specific IgE.

3.3 Immunoblotting and immunoelectrophoresis

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), p*I* (IEF) or both (2-DE), or combinations of physicochemical and immunochemical characteristics (crossed immunoeletrophoresis, 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 serum from allergic subjects 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 radioimmunoelectrophoresis (CRIE) method, where an IgE-immunoradiometric assay is performed to visualize the IgE-binding capacity of precipitates in a CIE-gel. A comparative study has suggested that the optimum result is only obtained by a combination of the different methods [61]. Although less quantitative, it is also possible to perform the immunoblotting method as inhibition assay [62] in order to confirm the specificity of an obtained result and also to identify (by the molecular weight and/or the p*I* or known identity of a purified inhibitor) the detected protein(s). Sensitivity and overall results of immunoblotting and immunoelectrophoresis are strongly influenced by experimental conditions such as amount of protein subjected to analysis, electrophoresis and transfer conditions, blocking procedures and other factors. For each combination of antigen and detection reagents, unspecific binding of detection reagents has to be excluded by appropriate control experiments.

3.4 Basophil histamine release (HR) tests

The starting point of a type I allergic reaction is crosslinking by allergen of specific IgE bound to high-affinity receptors on effector cells, *i.e.*, mast cells and basophils. Efficient crosslinking will trigger release of mediators such as histamine. This process is dependent on the affinity of the interaction between IgE and allergen and the valency of this interaction [63]. For crosslinking, a minimum of two epitopes is needed, but it has been established that >2 epitopes is more efficient. The minimal threshold for IgE affinity and avidity has not yet been elucidated. It is also not really clear whether a single high-affinity interaction combined with one or more low-affinity interactions are sufficient for effector cell activation. Possibly, in poly-sensitized patients, simultaneous recognition of multiple allergens has a synergistic effect leading to efficient mediator release at lower level of avidity. *In vitro* tests for measuring the biological activity of IgE antibodies have been available for several decades now. In contrast to mast cells, human samples enriched for basophils can easily be obtained. For this reason, most assays are being performed with basophils instead of mast cells, although the mast cell plays a more dominant role in immediate type allergic reactions. In most cases histamine is measured in cell supernatants by various detection methods [64–67], but also other mediators like leukotrienes are analyzed [68]. A more recent development has been to detect the expression of basophil activation markers like CD63 [69, 70] and CD203c [71] instead of measuring the release of mediators.

Since some allergen extracts have been reported to contain histamine [72] it is essential that the histamine content of each preparation of test protein is checked. 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.

Theoretically, the clinical relevance of these tests for biological activity is expected to be higher than of assays that simply measure the presence or absence of specific IgE antibodies, and several studies have indeed shown a higher specificity and better correlation to the clinical situation (EAACI position paper "Biological allergy tests" Poulsen *et al.* (in press)). Receptor-bound IgE antibodies that do not mediate HR or increased expression of CD63 upon incubation with the appropriate allergen, most likely will not cause clinical symptoms. In the dispute on the clinical relevance of IgE-binding *N*-glycans, basophil HR tests have been used to demonstrate possible involvement of these cross-reactive structures in clinical food allergy [62, 73–75]. In some cases, quantities of allergen needed to induce a biological effect were in the 100 ng/mL–10 mg/mL range. For strong allergens with accepted clinical relevance HR is frequently achieved at concentrations between 100 pg/mL and 10 ng/mL [76, 77]. Similar differences were observed between the potent areoallergen, Bet v 1 from birch pollen, and the homologous apple allergen, Mal d 1 which is known to cause mainly mild oral symptoms [78]. It can not be ruled out completely that part of these differences were caused by

different protocols used for assessing biological activity (*e.g.*, direct release from patients' basophils *versus* stripped and resensitized basophils). Further analyses will have to elucidate whether an allergen threshold for biological activity can be established below which IgE antibodies are likely to be of clinical importance. To this end, subjects with food-specific IgE with and without a positive DBPCFC will have to be compared by basophil activation tests, and method validation studies will have to be performed.

For routine diagnosis, cellular tests are less convenient because fresh cells of the patient are needed. To overcome this, a protocol was developed that uses basophils from non-allergic volunteers that are stripped from their IgE with lactic acid [79]. Subsequently, these cells can then be resensitized with serum IgE from allergic patients. This method allows evaluation of multiple patients with cells from a single donor. Alternatively, cell-lines like rat basophilic leukaemia (RBL) cells transfected with the human alpha chain of the high-affinity IgE receptor have been created [80, 81], allowing sensitization with human IgE. The advantage is that these cells can be cultured permanently, making the biological test even more flexible, independent from availability of human basophils, and facilitate an improved standardization of the method. However, the potential of these tests in food allergy diagnosis needs to be investigated.

For the assessment of allergenicity of novel foods, a biological test is of utmost importance. In cases where serum samples or whole blood samples from DBPCFC-positive subjects can be obtained, these materials should be used for evaluation of allergenic activity. However, it is not always feasible to obtain serum samples from DBPCFC-proven allergic patients for RAST-type analysis to assess potential allergenicity. Especially in these cases, validated biological tests might in the future provide relevant information on the potential clinical meaning of IgE binding to novel foods or heterologous proteins expressed in genetically modified plants.

4 Selection and quality of sera

4.1 Selection of sera for specific serum screening

The WHO/FAO 2001 guidelines (Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/y0820e/y0820e00.htm) and the Codex 2003 document (Codex Alimentarius Commission, 2003, Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents) underscored 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 titres, it is important to employ high titered sera from patients evaluated according to international guidelines, such as the newly published European Academy of Allergology and Clinical Immunology guidelines for DBPCFC [82]. If the protein in question does not constitute a major allergen (*i.e.*, an allergen to which more than 50% of an allergic population react), a large number of sera may be necessary to achieve sufficient certainty that an allergenic protein may be identified, *i.e.*, individual sera from 24 patients will assure that an allergen to which more than 20% of the population react will be detected with 99% certainty (Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/y0820e/y0820e00.htm).

Since the publication of the WHO/FAO guidelines in 2001, only a few studies have been trying to adapt them. In one study [83], a protein derived from fish was tested for homology with known allergens without finding any. Subsequently, the authors selected sera from 20 fish allergic patients, most of which tested positive to the source of the new protein. When the protein itself was tested, it consistently produced negative results in both RAST and HR, indicating that none of the 20 fish allergic patients had reactivity to that particular protein [84].

4.2 Selection of sera for targeted serum screening and further characterization of homology between a new protein and a known allergen

The WHO/FAO 2001 guidelines (Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/y0820e/y0820e00.htm) considered the case where no homology to a known allergen has been identified and where it may be relevant to test sera with a known reaction to allergens coming from the same class of allergenic source, such as yeast/molds (patients allergic to spores from Alternaria or Cladosporium or with aspergillosis or Trichyphyton sensitivity), monocots (wheat and rice), dicots (tree or weed pollen, celery, peanuts, tree nuts, and latex), invertebrates (mites, cockroach, shrimp, chironomids, and silk), vertebrates (mammalian pets, laboratory animals, milk, and egg), and others (with only the negative notion that no general screen is available). It was suggested to use 25 sera with reactivity to aeroallergens and 25 with reactivity to food allergens. Since a scientific justification or validation of this approach was lacking, the Codex 2003 document (Codex Alimentarius Commission, 2003, Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents) recommended the target serum

screen as a future tool that might be included in the assessment after further evolvement of scientific knowledge and technology.

Concerns about the usefulness of targeted serum screening are mainly related to specificity of the result in relation to the clinical situation: It is not exceptional that IgE antibodies against foods are detected in patients who do not present symptoms of clinical food allergy. This phenomenon of clinically false-positive test results is in particular observed in patients with atopic dermatitis who have very high total IgE levels, and in pollen allergic patients [7, 11–13, 19, 42]. IgE antibodies against highly conserved allergenic structures like pollen *N*-glycans [20–23] and profilin [12] are common among these patients and are crossreactive to plant foods. In studies that reported clinical relevance of profilin or *N*-glycans, patients were usually selected on the basis of clinical food allergy instead of pollen allergy [73, 75]. Thus, in the average pollen allergic patient, sensitization to these conserved structures is likely to result in clinically insignificant crossreactions rather than clinical food allergy.

Among food allergens, there certainly is a spectrum from very rare association of IgE binding with clinical symptoms to allergens for which a positive IgE assay indicates a substantial risk of a life-threatening reaction. When the food allergen itself is at the basis of sensitization, the chance of being involved in (severe) clinical food allergy is most likely higher than when its recognition by IgE is a result of crossreactivity, because a higher avidity can be expected for the food-specific IgE antibodies. IgE antibodies against the major peanut allergen Ara h 2, for example, are induced as a result of peanut consumption and are thought to be of major importance for peanut allergy [85]. In contrast, IgE antibodies against Ara h 5 (peanut profilin] are a result of pollen sensitization and show little or no correlation with clinical food allergy [12]. Generally speaking, clinical relevance of crossreactive IgE antibodies decreases in parallel with the phylogenetic relationship between primary sensitizer and crossreactive food. The explanation of this phenomenon has to be found in the affinity and valency (avidity) of the interaction between allergen and IgE [63]. Both will decrease together with a decline in structural homology (phylogenetic relationship). Moreover, it has been suggested that stability of the food allergen recognized by IgE, in particular under influence of the proteolytic environment of the gastro-intestinal tract, is another factor influencing the correlation between detection of specific IgE antibodies and clinical food allergy [86]. Stable allergens like LTP and 2S-albumin are more likely to induce severe systemic reactions than labile allergens such as profilin or Bet v 1–related food allergens. The outcome of the interaction between IgE and food allergens is therefore dependent on a complex interplay between IgE affinities, epitope spectrum and physicochemical characteristics of the allergen. Taken together, these considerations have several implications for the selection of sera for targeted serum screens, in particular if not only pure proteins, but whole novel foods are tested:

(i) Similar to the specific serum screen, sera should be selected primarily on a clinical basis.

(ii) Individual sera are preferred over pools.

(iii) If possible, the recognition by IgE of individual allergens in the allergy-eliciting material should be determined.

(iv) Sera from subjects with atopic dermatitis and very high total IgE levels should be excluded from serum screens if no evidence for type I reactions exists.

(v) Sera with IgE antibodies to complex N-glycans should not be used for screening of crossreactive sources, unless the contribution of theses antibodies to the test result can be eliminated.

(vi) The presence of IgE against other highly crossreactive, ubiquitous structures such as proflins or Bet v 1 homologs should be noted and positive results interpreted with caution. Confirmation of the clinical meaning of the results is required. Even in subjects with proven food allergy, a positive test result does not necessarily mean that all antibodies are able to trigger allergic symptoms.

In the case where a bioinformatics search has suggested some degree of one or more common epitopes between a known allergen and a new protein, the putative crossreacting allergens should be considered the target, and sera expressing reactivity against these allergens should be used for further screening. If IgE-reactivity was only determined with allergen extracts containing a number of different allergens to which a serum may react, it should be established or at least qualified that a given serum actually reacts to the relevant allergen, in order to improve sensitivity of the system.

A risk evaluation has been carried out on a bacterial transglutaminase isolated from the organism *Streptoverticillium mobaraense* [87]. Even though it is not a GMO-product, the WHO/FAO 2001 guidelines were used as a basis for its evaluation [88]. Transglutaminase is an enzyme widely distributed in nature and can be found in diverse animal tissues, fish, and plants [89]. Microbial transglutaminase has no homology with transglutaminases found in plants and animals [90]. It has a molecular weight of 38 kDa and contains no saccharide or lipid moieties [91]. When testing the primary sequence, a match was found at the five contiguous amino acids level between m-TG and the allergen Gad c 1. Due to the fact that Gad c 1 is the major codfish allergen and extremely heat stable [92, 93], it was decided to investigate a possible crossreactivity between codfish and micro-

Table 1. Proposal for classification of sera available for screening of new proteins

Classification	Patient characterization	Comment
AAA	[82] Clinically reactive patient	Food allergens: Patients with a state-of-the-art diagnosis of food allergy (such as the EAACI guidelines [82]) to the allergenic food in question. Inhalation allergens: Patients with a convincing history of clinical reactions upon exposure to the allergenic source, supported by positive specific IgE, SPT, or HR test to an extract of the allergenic source
AA	Anecdotal clinical reaction	Patients with a case history that has not been confirmed. This may be supported by positive skin tests and/or specific IgE against the food in question
A	Only IgE-positivity known	Sera for which there are no clinical data, but only positive IgE-determinations (CAP, RAST).
0	Random serum screen	Sera from population-based studies, chosen to represent a typical profile of IgE-reactivity in the general population or a selected subpopulation.

bial transglutaminase, and sera from 25 documented cod fish allergic patients were included. We have previously found that IgE reactivity to Gad c 1 is a prominent feature of clinically allergic patients [94], and no further attempts were made to characterize the individual IgE-binding pattern. Upon testing no crossreactivity was observed between codfish and transglutaminase [88].

In the selection of sera, less than perfectly well-characterized sera may be the only available. A proposal is given in Table 1 for a classification of sera depending on their degree of verified clinical diagnosis. Whereas sera showing positive reactions to a food or an inhalation allergen may be used for studying the degree and the character of crossreactivity between a known allergen and a new protein, it is suggested – and in accordance with the guidelines (Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/y0820e/y0820e 00.htm) (Codex Alimentarius Commission, 2003, Food and Agriculture Organization of the United Nations, FAO Corporate Document Repository, http://www.fao.org/documents) – that only sera from clinically reactive patients (AAA) are actually used to perform risk evaluations. As indicated other classes of sera may still be interesting and relevant for outlining the type of crossreactivity between a known allergen and a new protein. However, one has to be aware that even sera from AAA patients may contain IgE antibodies of low biological activity.

5 Method validation and control experiments

Two levels of method validation have to be distinguished:

(i) The validation of any IgE immunoassay as an analytical tool.

(ii) The validation of serological results in relation to the clinical situation.

To the first level, international guidelines for method validation apply, of which single laboratory validation represents the lowest level [95]. Ideally, method performance criteria such as intra- and interassay precision, sensitivity, specificity, robustness, *etc*. should be determined in ring trials in accordance with international standards (International Standard ISO 5725 (1994)) [96]. In addition, an appropriate number of nonallergic controls and high IgE controls (*i. e.*, sera from subjects allergic and IgE-positive to noncross-reacting allergenic sources) should be included. The second level of validation has been discussed extensively in the previous sections of this article. As outlined, each positive result in an IgE test requires further investigation of its clinical meaning. The second step of the evaluation should be confirmation of biological activity by mediator release tests which, in case of a positive result, should be followed by DBPCFC, before conclusions on allergenicity can be drawn. This leads to the following hierarchy of tests:

(i) IgE binding

(ii) Basophil HR

(iii) Challenge test (DBPCFC)

6 Concluding remarks

Diagnostic tests for food allergy based on measurement of specific IgE frequently suffer from low sensitivity and/or specificity in regard to predicting the clinical situation. The introduction of well-characterized purified major allergens is a significant step forward in the improvement of specific IgE tests for food allergy. Nevertheless, assessment of biological activity will also be needed to distinguish biologically inactive from active IgE antibodies. Similarly, assessment of potential allergenicity of novel foods should not be based exclusively on the detection of IgE binding. It is important to avoid designation of a novel food as an allergenic risk without having assessed the biological activity of established IgE binding. However, thorough validation of

biological allergen assays in relation to the clinical situation for the majority of important food allergies is required.

What can be deduced from a positive or negative result in IgE-screening of a novel food protein? Besides the fact that negative testing in a specific serum screen strongly suggests that the protein under study is not an allergen, the outcome of the testing of a new protein against sera with IgE-reactivity to known allergens or allergenic materials is far from fully predictive of the potential clinical reactivity of patients toward the protein in question. Important pitfalls include:

(i) Even though studies have indicated that there is a rough correlation between the concentration of specific IgE in serum and outcome of DBPCFC, a number of studies seem to indicate that the cut-off value may be highly dependent both on the allergen in question and the investigated population [57–60]. Likewise there is little evidence that the threshold dose, *i.e.*, the lowest dose to which patients react, could only be predicted by the IgE titer.

(ii) In many cases, a number of well-characterized sera were not available for a statistically robust investigation of a new protein. As discussed above, the scarcity applies both to the quality and the number of available sera. If a new protein crossreacts with a major allergen in a known allergenic source, the testing may be feasible, but if it is a minor allergen with only 10% of clinically reactive patients testing IgE-positive, the number of sera necessary to perform the validation may become unachievable. Moreover, differences between populations, and different levels of exposure caused by nutritional habits have to be taken into account.

(iii) We may not be looking in the right place or using the right tools. The concept of serum screening could be seriously biased by our present understanding of allergens, or a research bias in what allergens have been described and cloned so far. Formation of large allergen databases will increase our knowledge, and perhaps the concept of common antigenic/allergenic epitope motifs [97] will replace the lists of allergenic foods as candidates for screening.

(iv) Features other than the (primary, secondary, or tertiary) amino acid sequence may determine the allergenic potency. These may include stability in general, resistance to degradation in the digestive system, or even be related to other components in the food which may alter the absorption of an allergen, and thus rendering it more or less allergenic, than what would appear from a purely *in vitro* assay employing the purified protein.

As discussed above, even the identification of IgE-reactivity does not necessarily mean that a new protein will be an allergen in humans because the result may be caused by crossreactivity involving IgE antibodies with low affinities, and thus low biological activity. In this context, the usefulness of targeted serum screening, which may favor the detection of low-affinity, crossreactive antibodies, needs to be further evaluated before this approach can be generally recommended. Therefore, as a practical approach, risk evaluation procedures including limited and very focused IgE testing may eventually be developed, which will allow the estimation of the allergenic risk of new proteins in relation to those already present in the global marketplace for foods. However, it has also to be noted again that any kind of serum screening is incapable of providing an assessment of the sensitizing capacity of a given novel food. Since the majority of allergenic protein families appear to be known [30, 98], it is likely that concepts like "the big eight", *i.e.*, the proposed list of most common foods to elicit allergies, might in the future be complemented by lists ranking individual proteins or protein families on the basis of the known allergenic potential both *in vivo* and *in vitro*.

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