

In Vitro Diagnostic Methods in the Evaluation of Food Hypersensitivity

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KEY CONCEPTS

- There are validated *in vitro* markers for food allergy diagnosis.
 - Specific IgE antibodies in case of IgE-mediated allergy.
 - Specific IgA/IgG antibodies to gliadin and endomysium in case of celiac disease.
- More information is obtained if markers are evaluated quantitatively.
 - Information both regarding the risk of a reaction and of possible outgrowth can be achieved.
- Cross-reactivity between different allergens needs to be taken into account.
- Evaluation of allergen components, epitopes, and genetic markers are promising for providing increased information in the future.
- It is important to use a technically well-validated and clinically evaluated laboratory system to achieve useful information.

Introduction

The technical feasibility and clinical utility of *in vitro* determinations of antibodies and other markers will be discussed with specific emphasis on food allergy. Allergic diseases including reactions to foods represent an increasing problem in the Western world, with symptoms that may not be easily distinguished from other disorders. In this chapter, the term “hypersensitivity” is defined as something that induces objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal subjects [1]. Hypersensitivity can be differentiated into allergic hypersensitivity which involves an immune mechanism and non-allergic hypersensitivity where immune mechanisms are excluded. Different tests must be applied to distinguish between such conditions. An allergic hypersensitivity can be either IgE mediated or non-IgE mediated; an example of non-IgE-mediated hypersensitivity is celiac disease (CD), which involves immune cells and antibodies of IgG and IgA isotypes. In distinguishing between the two types of allergic hypersensitivity, a given test should identify

the IgE- or IgG/IgA-related allergic mechanisms in allergic patients from those of other patients suffering from similar symptoms.

When suffering from allergy, most patients are sensitized to more than one allergen that may trigger their clinical symptoms. It is often difficult to distinguish which is the clinically responsible allergen. Furthermore, allergic symptoms related to IgE antibodies depend not only on those IgE antibodies, but also on a number of related and unrelated confounding factors. These factors include inflammation, organ function, presence of infection, physical and psychological stress, and hormonal influences and the IgE antibody results must always be interpreted in context of these confounding factors. For patients with food allergy and intolerance, the double-blind, placebo-controlled food challenge (DBPCFC) is considered the gold standard [2,3]. However, this technique does not distinguish among allergic hypersensitivity involving IgE antibodies, antibodies of IgG/IgA isotypes, and cellular immune mechanisms, and those of intolerance, including enzyme deficiencies or other unknown mechanisms exhibiting similar degree of hypersensitivity.

A clinical diagnosis of IgE-mediated allergy should be based on the patient's history, symptoms, findings on physical examination, together with laboratory test results. Diagnosing patients with IgE-mediated allergy would differentiate them

from those having several other disease etiologies presenting with similar symptom profiles. Because there is no working gold standard for true allergy diagnosis, only one for food hypersensitivity and CD, such diagnosis is complicated. With the recognition that the prevalence of allergic problems is increasing and differential diagnosis by history and physical examination is difficult, tools should be used to differentiate the mechanisms behind the symptoms. The present communication discusses well-defined blood tests for specific IgE or IgG/IgA antibodies and other markers, representing objective means to identify food-specific allergies in individual patients. The presence or absence of such antibodies or markers can be determined with high sensitivity and precision. Such information represents one piece of information among others that must be used to compile a definitive clinical diagnosis.

Antibodies to various allergens may be present without obvious clinical disease. Nevertheless, the presence in a very young child of minute levels of specific IgE antibodies, especially to hen's egg white and, to a lesser extent, cow's milk, can be used as a predictor of evolving sensitization to inhalant allergens and allergic disease [4]. In contrast, the presence of IgG/IgA antibodies to a specific food may just be the result of exposure to the substance or allergen, sometimes associated with increased permeability of the gastrointestinal mucosa, but without an obvious link to a clinical disorder [5–7]. Exceptions are the IgG/IgA antibodies to gliadin as well as tissue transglutaminase (tTG) in CD [8] (Box 19.1).

For markers of inflammation, the situation is less consistent and sampling is often problematic. However, there are methods for determining markers from (1) mast cells, for example histamine, tryptase, prostaglandins, and leukotrienes [9–11]; (2) eosinophils, for example the eosinophil granular constituents such as eosinophil cationic protein (ECP), eosinophil protein X (EPX), eosinophilic peroxidase (EPO), major basic protein (MBP), and leukotrienes [9–11]; (3) basophils, for example histamine and leukotrienes [9,10]; and (4) neutrophils, for example myeloperoxidase (MPO), human neutrophil lipocalin (HNL), lactoferrin, and lysozyme [11], although these are often not well established as clinical diagnostic methods but are to be considered more as research tools [9–11].

For conditions due to enzyme deficiencies, the situation is even worse, except for lactose intolerance, most tests to determine enzyme deficiency are less well proven. For example, in histamine intolerance mediated by a deficient diamine oxidase system [12], standardized methods have not been established, despite the fact that the clinical condition is recognized and often presents as headache after ingestion of certain histamine-, phenyl ethylamine-, or serotonin-containing foods such as red wine. Since the enzyme is located primarily in the jejunal mucosa, the problems are induced by gastrointestinal exposure. The diagnostic tests for this condition are of a research nature and are difficult to use in clinical practice [12]. However, recently there have been promising reports suggesting new methods to diagnose such conditions as irritable bowel syndrome by ultrasonic sound, focusing on local edema in the intestinal tract [13,14], so new diagnostic methods for other disorders are likely to be developed.

Markers and methods with confirmed value

IgE antibodies

It is well known that the presence of IgE antibodies to a specific food indicates a certain probability of a clinical reaction to that food, although the risk levels are unique in each patient. Virtually any food may lead to a reaction, although only a small number of foods, such as in children where hen's egg, cow's milk, peanuts, soy, wheat, tree nuts, and fish account for about 90% of the reactions [15–17]. In adults, peanuts, tree nuts, fish, and shellfish are the most common, although sensitivity to other allergens may be present and must be identified by case history together with allergy testing [18–20]. Negative results obtained with *in vivo* tests and skin prick tests (SPTs) are informative with a very high negative predictive value (NPV) (>95%) [21,22]. When interpreted as a positive result, allergy tests show great variations in clinical specificity for different allergens in different patient groups. Evaluating the quantity of IgE antibodies, rather than using results in a simple dichotomous manner, has been shown to provide more information. The correlation of the quantity of allergen-specific IgE and the probability of clinical reactivity were first demonstrated by Sampson and co-workers [23,24] and later confirmed by other investigators [25–32]. However, it is important to emphasize that the age of the food-allergic patient affects the interpretation of the level of the IgE antibodies [25,26,29,33]. Utilizing the fine-tuning of IgE antibody specificities to different epitopes may in the future provide an even better predictor of which patients will experience allergic reactions when eating a food, rather than just being sensitized [34] (Box 19.2).

Monitoring IgE levels also may be clinically important in the follow-up of patients with allergy to food. For example,

Box 19.1

Case history, physical examination + laboratory tests = clinical diagnosis

Presence of markers + clinical symptoms = possible clinical disease

Presence of markers + no clinical symptoms = may suggest ongoing disease or outgrowth of disease

Box 19.2**Most frequent allergies**

Children		Adults	
IgE	IgG/IgA	IgE	IgG/IgA
Hen's egg	Wheat	Peanut	Wheat
Cow's milk	Gliadin	Tree nuts	Gliadin
Peanut	Endomysium	Fish	endomysium
Soy		Shellfish	
Wheat			
Tree nuts			
Fish			

in patients destined to “outgrow” their allergies to cow's milk protein, the levels of IgE antibodies to cow's milk proteins were found to be lower than in those patients with persistent allergy [35–37]. The antibody specificity and especially the diversity of antibody specificities also seem to impact the patient outcome [38,39].

IgG/IgA antibodies in celiac disease

The etiology of CD is yet to be fully resolved. It involves an autoimmune enteropathy triggered by the ingestion of gluten in susceptible individuals. Even though CD is usually considered to present in early childhood, only a minority of patients are diagnosed during this time, while the vast majority of CD patients are diagnosed much later or not at all. The typical intestinal damage, such as loss of villi and hyperplasia of crypts, resolves completely upon elimination of gluten from the diet. Besides failure-to-thrive, a large proportion of CD patients present with atypical symptoms such as general weakness, bad temper, anemia, menstruation disturbance, or even depression.

Historically the diagnosis of CD has included the need for several intestinal biopsies as outlined in the first European Society of Pediatric Gastroenterology, Hepatology, and Nutrition guidelines [40]. Since the development of more sensitive and specific *in vitro* tests for specific IgA and IgG antibodies to gliadin, endomysium, and tTG, the guidelines have been revised and the number of biopsies needed to ascertain a valid diagnosis has been reduced [41]. The combined use of IgA and IgG anti-gliadin antibodies (AGA) measurements available in different *in vitro* assay formats (e.g. enzyme-linked immunosorbent assay, ELISA; ImmunoCAP) has been shown to be sensitive and specific in relation to the presence of clinical disease [8].

Histochemical staining methods used to detect IgA antibodies to endomysium have been considered more specific and sensitive, but also more difficult to perform in a standardized manner. The demonstration that tissue tTG is the main target of the autoimmune response [42] has led to the development of several specific IgA assays based on guinea

Box 19.3

The most important antigens in CD	Antibodies
Gliadin	IgA/IgG in cases of IgA deficiency
Tissue transferase	IgA/IgG in cases of IgA deficiency

pig or, preferably, human tTG, giving very high sensitivities and specificities. Tests with recombinant human tTG seem to perform the best and are a useful tool in both small children and adults [8,43–46]. In the cases of IgA deficiency, specific IgG antibodies to gliadin and tTG are of special value [47,48].

The presence of serum tissue tTG and endomysial autoantibodies is predictive of small-bowel abnormalities indicative of CD. There is a good correlation between autoantibody positivity and specific human leukocyte antigen (HLA) haplotypes. Although population screening for CD using serological tests is still a controversial topic, screening of high-risk groups, such as first-degree relatives of CD patients, patients suffering from osteoporosis, anemia, type I diabetes, thyroiditis, IgA deficiency, or other autoimmune diseases, is strongly recommended [49–51] (Box 19.3).

Allergen and antigen properties in the *in vitro* diagnosis**Allergen sources**

The ability of a test to detect specific IgE and other antibodies depends on the presence of all relevant allergen components in the test system. Food is prepared from both animal and plant origin and the antibody patterns of patients sensitized to food are often even more complex than those seen for inhalant allergens. The use of native allergen source material of the highest quality and containing all relevant allergen components is of primary importance. The mode of preparation of extracts for preservation of allergenic potency during processing is vital [52–54].

Intact macromolecules from the partly digested food may pass through the intestinal mucosa into the circulation and act as allergens [5–7]. Attention has also been drawn to the possible creation of neo-allergens during processing and digestion of food; for example, the allergenic determinants are enhanced and/or formed by roasting peanuts [55,56]. In some cases, food allergens are destroyed during processing, as exemplified by the fact that some patients may tolerate the cooked food, but not its raw counterpart [57,58]. To be able to detect all patients with differing antibody specificities, a native food source material that is representative of the natural exposure should be used. Fermented food may have a lower content of effective allergens or exposure of such allergens to the gastrointestinal tract [59,60].

Box 19.4

Exposure to food allergens

Preparation of food

Raw

Boiled

Roasted

Dried

Fermented

Geographical differences

Ethnic differences

IgE antibodies are produced as a consequence of exposure to allergens in the diet and environment. Regional differences in food habits may result in different patterns of IgE antibody specificity [53,61–64]. However, increasing international trade, and use of tropical food, ornamental plants, and herbs widen possible exposure far from what is common in the local environment.

Currently, considerable research efforts are directed at characterization of individual food allergen components. Today more than 70 food allergen components are listed by the International Union of Immunological Societies (IUIS) (<http://www.allergen.org>) and many more components are described in the Allergome database (www.allergome.org). Many of these components have been cloned and may in the future be available as recombinant proteins [65]. The use of separate components or combinations of components may lead to new and better tools in the diagnosis of food allergy in the future and already today the increasing knowledge of reactivity patterns to different components can be useful in clinical practice [66,67] (Box 19.4).

Epitopes

Clinical sensitivity to a certain food allergen often changes over time. It is estimated that about 80% of children outgrow their cow's milk allergy [18], although only 20% outgrow their peanut allergy [68]. Some results suggest that IgE antibodies from individuals with persistent allergy may be directed against different epitopes than those in patients with transient allergy [34,38,39,69]. Epitopes may be continuous (linear or sequential) or conformational (involving different parts of peptide chains due to folding on the peptide chain), and the specificity of an antibody depends on the uniqueness of the epitope. The measurement of specific IgE to single epitopes may provide a new way of not only diagnosing, but also predicting allergic reactions in food-allergic children. In the future we may see tests identifying antibodies to different epitopes and then predicting whether the allergy is transient or persistent. Newer information also suggests a possibility to reveal the risk for anaphylaxis by utilizing the spectrum of IgE specificities to different epitopes on the same allergen molecule [38]. To obtain

such information by monitoring epitope-specific IgE antibodies over time, the test system needs to be quantitative and give correct results over the whole measuring range. The development of the multiplex technology platform would facilitate the use and interpretation of IgE antibody-binding patterns to define multiple components and epitopes [38,70,71].

Antigen sources

Gliadin, tTG: The common antigen source for determination of gliadin-specific IgA and IgG antibodies is crude or purified fractions of wheat gliadin. Gliadin is obtained as the alcohol-soluble fraction from wheat gluten prolamins. Prolamins from other closely related cereals such as rye, barley, and oats show some degree of cross-reactivity but are not commonly used for CD diagnostics [72,73]. For measurements of IgA and IgG antibodies to tTG, guinea pig-derived antigen was used initially, but human tTG has been shown to give higher sensitivity and specificity [8,44,73,74].

Cross-reactivity

Cross-reactivity between allergen-specific IgE and related allergens *in vitro* is also seen with SPTs. Consequently, the clinical relevance between different allergens must be determined individually for each patient, taking the clinical history and provocation/elimination diet results into account. Proteins with similar functions in different plant species may have a similar structure [75]. The IgE antibodies may detect such similarities between allergens from different sources as a function of biology and chemistry resulting in allergic reactions despite the fact that no apparent exposure to the allergen can be identified. Among foods there are several groups of cross-reactive allergens. The pollen-related food allergies to fruit and vegetables are well known, but cross-reactions have also been demonstrated between shellfish and other animals, between fruit and latex, and between different fruits [19,65,76–79]. The knowledge of cross-reactivities can be used for better assessment and thereby clinical management of the patients [67].

As discussed in Chapter 3, many food allergens from plant sources are proteins belonging to the “pathogenesis-related” (PR) protein family, for example, Bet v 1 homologs that have been identified in a great number of pollens and fruits [80,81]. Another group of PR proteins include the chitinases that are present in latex and fruits [80]. Other allergens known to induce cross-reactivity between pollens and fruits are profilins with highly preserved protein structures [82]. Lipid-transfer proteins (LTPs) compose another group of very stable proteins present in fruits and vegetables and cause cross-reactions both *in vitro* and *in vivo* [61,83–85]. In foods of animal origin, tropomyosins and serum proteins are known to be cross-reactive [76]. Future research on the basis of recombinant (or purified native) components

Table 19.1 Examples of common food allergens

Protein classification	Property	Allergen source (allergen)
PR-2	β -1-3-glucanases	Fruits, banana, latex (Hev b 2)
PR-3	Type I (basic) and Type II (acidic) chitinases	Avocado (Pers a 1), banana, chestnut
PR-4	Chitinases	Turnip, elderberry
PR-5	Thaumatococin- and osmotin-like proteins (antifungal)	Cherry (Pru av 2), apple (Mal d 2), bell pepper
PR-6	Protease and amylase inhibitors	Soy, wheat, barley, rye, rice
PR-9	Peroxidase	Wheat, barley
PR-10	Bet v 1 homologs similar to ribonucleases	Apple (Mal d 1), pear (Pyr c 1), cherry (Pru av 1), apricot (Pru ar1), hazelnut (Cor a 1.04), carrot (Dau c 1), celery (Api g 1), soy (Gly m 4), peanut (Ara h 8)
PR-14	non-specific lipid-transfer proteins (nsLTP), lipid metabolism	Peach (Pru p 3), apricot, plum, cherry (Pru av 3), apple (Mal d3), hazelnut (Cor a 8), maize, broccoli, carrot, rapeseed
Profilin	Actin binding, signal transduction	Celery (Api g 4), potato, hazelnut, apple (Mal d 4), pear (Pyr c 4), tomato, cherry (Pru av 4), soybean (Gly m 3), peanut (Ara h 5)
Parvalbumin	Ca ²⁺ -binding proteins	For example, cod (Gad c 1), carp (Cyp c 1), salmon (Sal s 1)
Tropomyosin	Muscle protein	For example, shrimp (Met e 1, Pen a 1, Pen i 1), lobster (Hom a 1), squid (Tod p 1), abalone (Hal m 1), scallop, crab (Cha f 1)
Seed storage proteins	Prolamin superfamily; 2S albumins, prolamins	Brazil nut (Ber e 1), walnut (Jug r 1), sesame (Ses i 1–2), cashew (Ana o 3), mustard (Sin a 1), rapeseed (Bra r 1), castor bean (Ric c 1), peanut (Ara h 2, Ara h 6–7), wheat (Tri a 19), rye (Sec c 20)
Seed storage proteins	Cupin superfamily; vicilins, legumins	Peanut (Ara h 1, Ara h 3, Ara h 4), soy, walnut (Jug r 2, Jug r 4), hazelnut (Cor a 9), vachew (Ana o 1–2), sesame (Ses i 3)
Protease	Proteolysis	Papaya (papain), pineapple (bromelain), fig (ficin), kiwi (Act c 1), soy (Gly m 1)

is needed to provide further information about correlations between structure and allergenic reactivity, and ideally will lead to the development of more specific tools for diagnosis. Table 19.1 shows some common food allergens.

Carbohydrate structures on glycoproteins may be involved in cross-reactivity between foods and pollens (cross-reactive carbohydrate determinants, CCD) [77,86,87]. Some of these carbohydrates have been identified and at least two important epitopes have been described that contain xylose and fucose [87]. An important and widely discussed issue is that IgE antibodies in a blood test may be bound to a univalent structure such as a carbohydrate, whereas biological activity, such as that shown in a skin test, may be negative because of the univalency of the test material [88]. However, this does not prove that clinical reactions will not occur when the individual is exposed to allergenic material containing the carbohydrates in a different multivalent conformation that can induce the biological activation of cells and mediators, triggering clinical reactions. Proteins carrying multivalent carbohydrate epitopes can induce histamine release [89], and these kinds of structures in some foods may be important in the clinical response [90]. Thus, it cannot be concluded that the IgE antibodies directed at carbohydrate structures are without biological and clinical significance [91,92] (Box 19.5).

Box 19.5

Common cross-reactivities

Plant	Animals
Pollen – food	
Bet v 1 homologs	Serum proteins
LTPs	Tropomyosin
Profilin	
Ca ²⁺ -binding proteins	
CCD (cross-reactive carbohydrate determinants)	

Performance characteristics of laboratory tests

Standardization of allergen and antigen extract in antibody tests

Extracts of allergen, antigen, or other markers of the inflammatory process used in allergy tests need to be standardized. These markers can be assessed with biochemical methods and/or by demonstrating antibodies in sera from known allergic individuals. Common methods include immunoblotting and inhibition of binding to the solid phase. It is of utmost importance to verify the reproducibility of different allergen batches produced. In particular, because the antibody specificities in

different patients are unique to various allergen components [93], the reproducible presence of all components on the solid phase of the assay system must be assured in order to obtain results relevant for a clinical interpretation.

Interactions between antibody and antigen

The immunological methods used to determine the presence and levels of antibodies and antigens in solution and on a solid phase matrix follow simple chemical rules. Many assays today utilize a solid phase for easy separation of reacted and non-reacted reagents. Similar chemical rules regulate the interactions between receptors on cells and their ligands. From the law of mass action applied to a heterogeneous solid phase immunoassay, it can be concluded that when the value of the allergen concentration multiplied by the equilibrium constant exceeds 10, more than 90% of the antibodies are bound and the reaction becomes antibody affinity independent [63]. Therefore, all allergen components in an allergen extract used in the method need to be in large excess to provide such high binding capacity for all antibodies regardless of antibody affinity and antibody class. A few commercial assay systems fulfill these criteria [94], which enable them to quantitatively measure all IgE antibodies present in serum samples without being distorted by background noise or inhibited by simultaneously binding IgG antibodies [95]. For instance, in two of the most extensively studied systems for IgE antibody determination, it was shown that 85–100% of the allergen-specific IgE antibodies present in allergic serum samples were bound to the solid phase surface [96]. Furthermore, using the same two systems, immunoblotting experiments revealed that all IgE antibody specificities present in a serum sample are similarly bound to the allergens on the solid phase, giving a representative quantitative result [96]. It is important to emphasize that such efficient binding of all relevant antibodies indeed is not true for all assay systems in use today [94,97]. In contrast, other systems have been proven to only determine antibodies of high affinity and failing to detect those with low affinity [98]. The consequences of such measurements are still unknown, however, and further studies are warranted before any conclusions can be made.

The test construction may impact this as was clearly demonstrated recently using chimeric antibodies [97]. In particular it has been demonstrated that the relative binding efficiency of the surface of a microwell used in many ELISA systems is too low to be able to pick up all antibodies. The reaction becomes affinity dependent, resulting in dilution curves that are not parallel; a true quantification is therefore impossible and gives results difficult to interpret [99]. Therefore, serum samples must always be diluted to reach optimal concentration conditions in such systems. For instance, IgA and IgG antibodies to gliadin and tTG can be accurately quantified in such systems after 100-fold dilutions. Table 19.2 shows examples of some test principles.

Calibration

Much effort has been focused on assays that can identify allergen-specific IgE antibodies because of their clinical importance in mediating immediate hypersensitivity reactions, including anaphylactic reactions, and their low levels in patients' sera. Since the first test for IgE antibody determination became available, there has been considerable development in the field. The original radio allergosorbent test (RAST), which became available in 1974, included a calibrator consisting of serial dilutions of a serum sample containing IgE antibodies to birch pollen. This was used to construct a calibration curve providing results in arbitrary units (Phadebas RAST unit/ml) and internally calibrated against the World Health Organization (WHO) International Reference Preparation for Human IgE 69/204 [100]. Newer generations of test systems usually replace the allergen-specific IgE antibody reference with a calibrator directly traceable to the WHO International Reference Preparation for Human IgE 75/502, which is one prerequisite for quantitative measurements of IgE antibodies [93]. In addition, specific absorption of antibodies should result in a parallel decrease of the content of total IgE [93,96,101]. The newer test technologies enable IgE detection at 0.1 kU_A/l, without jeopardizing the specificity of the test [102]. All test systems do not meet requirements for such sensitivity, however. The clinical relevance of detecting antibodies in such low range remains to be established, however.

For tests measuring IgG and IgA antibodies and other markers, development of calibration has been studied less extensively. However, several systems have applied calibration curves that provide determinations in relative units in a semi-quantitative manner and allow the comparison of results from time to time. Because there are no international reference preparations for allergen-specific IgG or IgA antibodies, the same concept used for specific IgE has also been used in some systems, that is, the use of a calibration curve consisting of total IgG or IgA for which there are WHO reference preparations available. The prerequisite for using this kind of calibration is that dilutions of samples are parallel to the calibrator curve in the system used. This approach can ensure stability and reproducibility over time.

Validation

For IgE antibody determinations, specific recommendations for performing tests have been published by the Clinical and Laboratory Standards Institute (CLSI) [103]. The recommendations include procedures for quality control for daily performance in clinical laboratory setting, and minimal performance targets of 15% coefficient of variation of IgE antibody assays. The College of American Pathologists has similar recommendations for IgG and IgA antibody determinations, as well as for determinations of other markers [101]. According to the guidelines by CLSI, a quantitative assay should meet criteria that include recovery of antibodies, precision, linearity and parallelism of dilution curves,

Table 19.2 Tests for discriminating the presence of allergy and tests for the identification of the offending allergen

Aim of the test to identify	Principle of the test	Basic technology	Major test system	Allergen coupling	Detection system
Presence of atopic condition	Multi-IgE antibody tests; for example, Phadiatop, including allergens from several different sources	Heterogeneous assay using a solid phase for separation of allergen-bound-specific IgE antibodies, labeled anti-IgE reagents	Phadiatop AlaTOP Allergy screen Multiscreen	Cellulose foam Soluble polymer Biotin-labeled allergen in solution Paper disk	Enzyme/fluorescence Enzyme/chemiluminescence Chemiluminescence Enzyme/absorbance
Presence of sensitization to specific allergens	IgE antibody tests to allergens from one source material	As above	ImmunoCAP Immulite 2000 Advia Centaur HY-TEC CLA-MAST	Cellulose foam Soluble polymer Biotin-labeled allergen in solution Paper disk Cellulose threads	Enzyme/fluorescence Enzyme/chemiluminescence Chemiluminescence Enzyme/absorbance Enzyme/fluorescence
Presence of sensitization to specific allergen component	IgE antibody tests to one single allergen component	As above	ImmunoCAP ISAC	Cellulose foam Glass slide μ -array	Enzyme/fluorescence Fluorescence
Presence of antibodies to specific antigens	IgA/IgG/IgG4 antibody tests to single antigens	Heterogeneous assay using a solid phase for separation of antigen-bound-specific Ig antibodies, labeled anti-Ig reagents	ELISA tests ImmunoCAP	Polystyrene Cellulose foam	Enzyme/absorbance/ fluorescence Enzyme/fluorescence
Presence of inflammation mediators from different cells	Histamine from basophils and mast cells	Solid phase with catching antibody, labeled anti-mediator reagents	RIA ELISA tests	Microparticles Polystyrene	Radioactivity Enzyme/absorbance/ fluorescence
	Tryptase from mast cells		ImmunoCAP RIA	Cellulose foam Microparticles	Enzyme/fluorescence Radioactivity
	Lipid mediators like leukotrienes and prostaglandins		CAST-ELISA ELISA tests	Polystyrene Polystyrene	Enzyme/absorbance Enzyme/absorbance/ fluorescence
	Eosinophil mediators like ECP, EPX, EPO		ImmunoCAP RIA	Cellulose foam Microparticles	Enzyme/fluorescence Radioactivity
	Neutrophil mediators like MPO, HNL		RIA	Microplates	Radioactivity
	Lymphocyte mediators like cytokines		ELISA tests	Polystyrene	Enzyme/absorbance/ fluorescence
Cellular immune response	T-cell proliferation	Cell cultivation with specific allergen/antigen stimulation and analysis of cell proliferation			

and calibrators over the measuring range. It states that all assay designs at the present time include a solid phase for separation of bound and unbound IgE antibodies, and all allergen components used must be in excess.

The question of whether different specific IgE antibody blood tests really give interchangeable results has been addressed. Proficiency testing is being undertaken in the United States by the College of American Pathologists, but is not being published and may be difficult to gain access to.

However, results from similar proficiency testing programs in Europe have been published. Those programs assess the performance of several commercial systems for the measurement of IgE antibodies specific to different allergens [104]. The testing indicated that the results from different assay systems are often not equivalent or interchangeable, although it has been demonstrated that some systems possess good performance characteristics [94,97,105]. In contrast, several other systems and assays do not meet

acceptable standards. However, such proficiency testing is more common for inhalant allergens than for food allergens.

Other tests of IgG and IgA antibodies and inflammation markers have been much less standardized. This makes comparisons between results obtained with different tests and methods more difficult.

Qualitative assessment

The qualitative performance of a particular test is usually evaluated in a clinical setting with a known population of individuals with and without disease and presented as the tests sensitivity and specificity. In such defined population, sensitivity is defined as the proportion of test-positive patients in relation to the total number of patients with the disease, whereas specificity is the proportion of test-negative persons in relation to the total number of individuals without disease. It is also common that the same known population and set-up is used to estimate the tests predictive value, that is, the proportion of test-positive patients in relation to the total number of patients positive by the test (Table 19.3) [106].

There is a considerable documentation for the presence of IgE antibodies in allergic disease, more than for many other test systems, and IgE antibody tests may therefore be taken as an example for the discussion. Thus, for IgE antibody tests, very good results of sensitivity and specificity have been documented for a variety of allergens [107]. Even for the early tests that were developed and marketed, data showed a good correlation between the levels of specific IgE antibodies and skin test reactivity or symptom scores [108].

IgE antibody test values of more than 90% for sensitivity, specificity, and predictive values have been obtained for certain test systems documented with several hundred patients [109]. However, it is difficult to relate the presence or absence of IgE antibodies exactly to the presence of clinical disease in an individual patient, especially because there is no absolute gold standard for IgE-mediated clinical food-allergic disease. Studies have confirmed the association between the levels of specific IgE antibodies and the degree of allergen exposure and development of symptoms [25–27,101,108–111]. Furthermore, in adults there may be clear-cut clinical evidence of food allergy without any detectable IgE antibodies [112]. In other situations, low levels of antibodies cannot easily be associated with clinical disease [23,24]. In CD, the gold standard is biopsy and clinical improvement with gluten-free diet [41].

When interpreting results from a dichotomized evaluation, it is crucial to carefully examine all conditions that the results are based on: the population used in the study, the number of subjects, etc. For all studies involving specific markers such as antigen-specific IgE, IgG, or IgA antibodies, it is also mandatory to specify the basis for defining a truly positive individual (Box 19.6), that is, to define the “gold standard.”

Table 19.3 Concepts in clinical validation of a test

		Test status		
		+	–	
Gold standard	+	A	B	A + B
	–	C	D	C + D
		A + C	B + D	A + B + C + D
True positive: A				
True negative: D				
Clinical sensitivity: A/(A + B)				
Positive predictive value: A/(A + C)				
Prevalence – Prior probability: (A + B)/(A + B + C + D)				
Efficiency – Concordance: (A + D)/(A + B + C + D)				
False positive: C				
False negative: B				
Clinical sensitivity: D/(C + D)				
Negative predictive value: D/(B + D)				

Quantitative assessment

Performance characteristics, like sensitivity and specificity, are necessary measures to demonstrate that a diagnostic test has the ability to discriminate between individuals with and without disease in a known population. However, when a quantitative marker is used, a dichotomization of the test result is a great over simplification. With the aim to decrease the number of time-consuming DBPCFCs for diagnosing patients with food allergy, Sampson [21,22] described the use of clinical “decision points” in a given food. He also applied a probability model for the same data, giving information regarding the decision points, and the important information indicating that even a low concentration of food-specific IgE antibodies may be associated with a certain – albeit low – risk of clinical reactivity.

The probability model is based on a logistic regression model relating the quantitative test result with the clinical outcome. The estimated relationship can be interpreted as the probability that a patient will react to an allergen as a function of the level of the specific marker, that is, the quantitative correspondence to the dichotomous positive predictive value. A low level will give a low probability for a patient to react and a high concentration will give a high probability for a patient to react (Fig. 19.1).

Different shapes of the relationship will indicate different identification patterns of symptoms; a steep curve indicates identification of symptoms even with low levels of the marker, whereas a flatter curve usually indicates that higher

Box 19.6

Qualitative approach

Dichotomous
Yes = presence; no = absence
of marker disease

Quantitative approach

Concentration of markers =
risk of disease

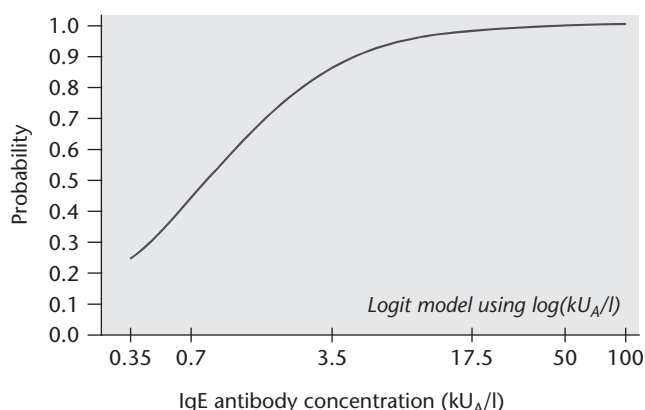


Figure 19.1 Representative logistic relationship between antibody concentration and the probability of reaction or obvious symptoms.

levels of the marker are required to make a diagnosis, or that no clear identification of the disorder can be attained with the test. Similar arguments can be made for IgA and, to a lesser extent, for IgG antibodies to gliadin and tTG and for other markers in which different concentrations result in different likelihoods of disease. The actual shape of these relationships must be studied for each individual marker.

Quantification of the marker and the use of logistic regression models can also be used for prediction of a likelihood that an event will occur. Shek *et al.* [36] used the decrease in specific IgE for hen's egg and cow's milk to predict the probability for a child to develop clinical tolerance. Their model would further improve if the time until clinical tolerance was included in the model, resulting in a higher probability for clinical tolerance if the decrease in specific IgE took place in a rather short time.

Compared with a dichotomous use of the decision points, quantification of the marker and the use probability models give more information about how the levels of antibodies are related to the likelihood of reactivity to food hypersensitivity.

Markers and methods with no confirmed value

Total serum IgE

Measurements of total serum IgE are used to give a very rough indication of whether there are any prerequisites of IgE-mediated disease in a patient. Given the considerable overlap between IgE levels in allergic patients and normal controls, and those with other disorders that may increase serum IgE (e.g. parasite infections), total serum IgE does not add considerable insight into the diagnosis of food allergy.

IgG/IgA antibodies in atopic allergy

There is no firm evidence that tests of IgG and IgG4 antibodies give any indication of the causes of clinical symptoms

in cases of immediate IgE-mediated reactions. Thus, IgG and IgA antibodies to foods are commonly found in both food-allergic patients and healthy persons. Such antibodies appear to be secondary to exposure to the food antigens/allergens and have not been shown to have any clinical value in the diagnosis of food allergy [113–117]. As an example, patients with CD often have high levels of IgG antibodies to cow's milk proteins. During the acute phase of the disease, they may have high levels of milk-specific IgG, and when the patient is in remission (after implementation of a gluten-free diet), levels decline [5,7]. However, recently there have been reports suggesting that IgG4 antibodies can have some association to delayed, non-IgE-mediated cow's milk allergy and in cow's milk-sensitive allergic eosinophilic gastroenteritis in children and young adults [118,119]. These studies were performed in patient groups rather than on an individual level and neither sensitivity and specificity nor probability for disease was given. Hence, it is too early to give any recommendation regarding such determination in the diagnosis of food allergy and more studies are warranted.

Histamine and basophil histamine test

Histamine released into the tissue and blood is gradually inactivated to methyl histamine. The relative amount of histamine and its metabolites over time following a clinical reaction is difficult to establish. Methyl histamine, which cross-reacts with histamine to some extent, can be determined by radioimmunoassay, although the half-life of both species is in minutes [120]. In the urine, histamine is not present, and methyl histamine or some other metabolite must be determined.

The basophil histamine test determines the release of histamine from peripheral blood basophils induced by cross-linking IgE antibodies bound to their specific cell receptors [121]. Also, complement activation and direct activation in some cases of idiosyncratic reactions to aspirin can release histamine. Given the difficulty of establishing optimum doses of allergens for release of histamine, and the difficulty of obtaining fresh blood cells, the test has been limited to academic and research settings. Development of whole blood semi-automated systems may, to some degree, circumvent the problem with high "spontaneous" basophil histamine release seen in food-allergic individuals who frequently ingest small amounts of the offending allergenic food [122]. A good correlation of this test method has been demonstrated compared to IgE antibody determinations in serum and to food challenges, although the results were never more predictive than IgE antibody determinations in blood or than SPTs [123]. When patients were compliant with diets excluding their offending allergenic food for several months, "spontaneous histamine release" decreases considerably [124]. In cases of *in vitro* challenges of peripheral blood cells with allergen, the results have been less conclusive. Furthermore,

about 10% of the population have non-responsive basophils that fail to release their histamine following allergen challenge *in vitro* [125]. The utility as well as the pitfalls of this test was recently reviewed [126]. Regardless of the difficulty in standardizing this test, the use of basophil histamine release may be warranted in particular situations using very well-controlled allergen components, for instance when anaphylaxis is suspected [126,127].

Tryptase, eosinophil cationic protein, and eosinophil protein X

Tryptase is found almost exclusively in mast cells. It has a much longer half-life in peripheral blood than histamine or histamine metabolites [128]. Unfortunately, in food-allergic reactions, the only situation in which tryptase determinations have been useful has been in some forms of anaphylactic reactions, where elevated levels have been documented in a minority of patients in both research and clinical practice settings [128,129]. However, the majority of fatal food-induced anaphylactic reactions where the tryptase level was measured in the peripheral blood demonstrated no elevation of plasma tryptase [130].

Eosinophil markers such as ECP and EPX in peripheral blood have also been used as research tools following food challenges in allergic individuals. Increased levels of these markers have been reported after positive challenges, sometimes in connection with decreased numbers of total eosinophils [131]. Recently, some investigators have been evaluating levels of EPX in the feces of patients undergoing challenges with suspected foods. It is still too early to determine the clinical usefulness of this procedure [132].

Granular constituents from eosinophils (ECP, EPX, EPO, MBP), neutrophils (MPO, HNL, lactoferrin), monocytes (lysozyme), and mast cells (tryptase) need to be isolated or cloned from human cells, because there is limited cross-reactivity between species [133].

Leukotrienes

Leukotrienes and, to some extent, prostaglandins have been used to monitor inflammation in allergic situations, mostly in patients with rhinitis, asthma, and drug allergy. Both LTE4 and the PGD2 metabolite 9 α ,11 β -PGF2 can be determined in the urine, although there is rather limited information on this in relation to food-allergic reactions [134]. A specific test, CAST-ELISA, has been developed to measure the leukotrienes LTC4, LTD4, and LTE4 released from peripheral blood cells [9–11,135]. Even if results have been reported from applications in food allergy, all these methods need further documentation of clinical correlations before they become widely applied in routine clinical evaluation. In particular, when allergens are applied to peripheral blood cells, care must be taken to avoid endotoxin contamination, which may give false-positive results [135].

Box 19.7

<i>Clinically confirmed markers</i>	<i>Clinically non-confirmed markers</i>
IgE antibodies in atopy	Total IgE
	IgG/IgA antibodies in atopy
IgG/IgA antibodies in CD	Histamine/basophil histamine test
	Tryptase, ECP, EPX
	Leukotrienes
	Cytokines

Cytokines

Serum cytokines have not yet proven useful in the clinical setting. This may be due to complexities related to the time they are obtained in relationship to the reactions and the different cells being activated [112]. Some studies have reported an imbalance of interleukin-4 (IL-4) and interferon- γ (IFN- γ) in children [136] and adults [137] suffering from allergic disorders. In infants with atopic dermatitis and cow's milk allergy, lymphocytes stimulated with allergen secreted high levels of IL-4, IL-5, and IL-13, in contrast to those tolerating milk who produced high levels of IFN- γ and very low levels of IL-4, IL-5, and IL-13 [138]. Other investigators have demonstrated that it may be of importance also to determine what cell population is producing the cytokines in the different conditions [139]. However, much more information is needed before such assays can be used in routine clinical evaluations. Table 19.2 shows various antibody and inflammation marker tests.

Tests to determine the T-cell response to gliadin in celiac patients expressing the genetic heterodimers of HLA-DQ2 or HLA-DQ8 may also be developed in the future [51] (Box 19.7).

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