

Standardization of *in vitro* methods

IgE-antibody analysis is a major diagnostic procedure and a primary tool in allergological research. The determination of sensitization frequencies and antibody concentrations against allergens of defined sources provides critical information for the estimation of the relative importance of food and environment in clinical allergy. True quantitation is essential and requires assay designs providing allergen excess and mass unit calibration. Standardized and reproducible methods show geographic and culture dependent differences between patient populations and contribute to the quality of diagnosis and treatment of allergic disease.

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A proper immunoassay for allergen-specific IgE antibody is designed to detect and quantitate all specific IgE in specimens that contain antibodies and to give a negative response for specimens that do not.

Performance standard

Very early data had already shown the correlation between allergen-specific IgE concentration and skin titration doses or symptom scores (1, 2). Later studies confirmed the correlation of IgE antibody concentration with the degree of exposure as well as with the severity of symptoms (3–5). Whether the presence of a detectable IgE antibody in a given patient at a given time leads to a reaction after natural or artificial exposure to the allergen is not always clear.

Attempts have been made to define the concentration of antibody that, with a specified degree of probability, can predict reactivity to a certain allergen (6). This is an interesting approach in the sense that it can be used as an indicator of cases for whom a challenge test would be a high risk and therefore could and should be avoided. However, it must not be understood as a measure of sensitivity of the immunoassay for specific IgE antibody. It is obvious that sensitized individuals may react, even strongly, at IgE-antibody concentrations much lower than the concentration corresponding to, for example, a 95% probability of reaction. All sensitized subjects are at risk and the statistical risk increases with increasing IgE antibody concentration.

To develop an immunoassay detecting a signal proportional to an antibody concentration is an immunochemical problem. To define the true presence

or true absence of a specific antibody is a clinical problem in the sense that there is no single independent reference method, *in vitro* or *in vivo*, that is free of variability caused by test material, patient selection (symptom, severity, age, environment, etc.), or investigator's interpretation.

Data from one selected patient group are, generally speaking, valid only for the group studied and the conditions under which the study was performed. Generally valid conclusions can only be drawn on data from studies where efforts have been made to compensate for the variations mentioned, i.e. by including representative patient selections (geographically, clinically), multiple criteria, and several expert views (7).

Assay design for ligand excess

Quantitative measurement of allergen-specific IgE antibodies against a specified allergen source, to indicate the degree of sensitization, requires the presence of an excess of all the allergenic components involved in the sensitization.

A high capacity solid phase provides conditions for quantitation of the sum of all antibodies regardless of specificity and affinity. The law of mass action, applied to heterogeneous solid phase immunoassays, says that when the available allergen concentration is increased to a level where K (the affinity constant) multiplied by the allergen concentration (eqn. 3) reaches ≥ 10 , the proportion of IgE antibody bound to the solid phase will be $\geq 90\%$ and essentially independent of the affinity between the antibody and the allergen (8, 9).

Law of mass action

$$\text{Association constant} = \frac{AB}{A \times B} \quad (1)$$

$$K = \frac{[\text{Allergen IgE Ab complex}]}{[\text{Allergen}] \times [\text{IgE Ab}]} \quad (2)$$

$$\frac{[\text{IgE Ab}_{\text{bound}}]}{[\text{IgE Ab}_{\text{total}}]} = \frac{K[\text{Allergen}_{\text{avail}}]}{1 + K[\text{Allergen}_{\text{avail}}]} \quad (3)$$

At a point where the assay is essentially affinity independent, i.e. when more than approximately 90% of the IgE antibody is bound, a further increase of the allergen concentration will have a negligible effect on the measured value.

Standardization and evaluation

Allergy tests are used to help in the diagnosis and monitoring of allergic patients. Assays need to be sensitive and specific and permit quantitative measurements over a wide range (10). As with all immunoassays, there is a need for common standards and definitions of performance parameters. In the case of total serum IgE, the WHO standard serves its purpose but for the measurement of allergen-specific antibodies there is no official standard collection of units and there are no uniform definitions.

In reality, however, Pharmacia CAP System[™] and UniCAP[®] have become a working standard against which all other commercial tests are compared. The calibrator chosen for these assays is a direct substandard to the WHO Reference Preparation 75/502 for IgE (11) covering the range 0.35–100 kU/l. Concentrations lower than the 0.35 kU/l calibrator are considered to be undetectable. Tests for all allergens are evaluated against this IgE standard.

Other commercial test kits largely adhere to this principle. An apparent agreement in terms of classes or units is, however, not always supported by data defining the true concentrations of the calibrators. Direct comparisons can therefore be difficult (12).

The IgE molecule has a number of epitopes on the Fc part. Combinations of catching-anti-IgE and conjugate-anti-IgE can be found that make the calibrator IgE and allergen-bound IgE appear identical. To what extent this applies to a certain assay can only be judged on the basis of experimental data and not on general assumptions. In the case of the Pharmacia CAP System it has been shown in model experiments with several allergens that the measurement of allergen-bound IgE is equivalent to IgE protein (13). This is possible because practically all the IgE antibodies of the sample are bound to the solid phase (14). For instance, in the case

of the timothy ImmunoCAP, the capacity to bind IgE antibodies was shown to be 25 times higher than what is required to bind all IgE antibodies at a concentration corresponding to the high end of the measuring range (100 kU_A/l). This is a large excess. Referring to the law of mass action it is clear that formation of allergen-IgE complex is favored. As mentioned, the excess is large enough to make the antibody binding independent of the association constant of the interaction (the affinity), regardless of the IgE antibody concentration. Furthermore, if all the relevant allergenic components of the source are present on the solid phase and shown to be capable of binding IgE antibodies, the system is capable of measuring the sum of allergen-specific antibodies and satisfies the requirements of a quantitative assay (15).

Quality control and documentation

Adequate controls should be included in every assay. Universally available IgE antibody control sera with defined concentration and allergen specificity are preferred over in-house pools. National and international quality control programs provide regular assessment of reagent and laboratory performance (16).

The basic quality documentation of an assay system includes:

- Quality and reproducibility of allergen source materials.
- Specificity and dose-response characteristics of labelled anti-IgE.
- Capacity and reproducibility of solid-phase binding.
- Calibration.
- Linearity and parallelism of calibrator and sample dilutions regardless of allergen.
- Recovery.
- Signal-to-noise ratio for the lowest calibrator.
- Freedom of interference.
- High-dose hook data.
- Stability of reagents and reagent combinations.
- Reproducibility.
- Correlation to reference method.
- Clinical sensitivity and specificity calculated on relevant patient populations.

Food allergens for specific IgE-antibody measurement

Early sensitizers

Intact macromolecules from partially digested food pass through the intestinal mucosa into the circulation. The IgE antibody patterns of food-sensitized patients are often even more complex than those seen for inhalant allergens. Egg white and cow's milk are the major causes of allergy in early childhood. IgE

antibodies in high concentrations appear during the first year of life. With time the antibodies and symptoms may decline and eventually disappear (17). Both sources contain heat stable and heat labile components and testing for antibodies to individual components or epitopes may aid in diagnostic and therapeutic decisions (18).

The early food allergy is often followed by sensitization to common environmental allergens and/or allergy to other foods. Local exposure to inhalants – like arthropods, animal pets, or pollens – and local feeding habits strongly influence the development of the disease. Selection of allergens for testing as well as interpretation of test results therefore must be based on local conditions and understanding of biological and chemical relations between allergens.

Nuts and fruits

Several frequently consumed seeds are commonly referred to as nuts or tree nuts, although only a few qualify for this botanical term. There are relations within this group, e.g. between walnut and pecan nut (*Juglandaceae*), but also between the food and inhaled allergens, e.g. hazel nut and birch pollen (*Corylales*). Unexpected cross-reactivity that can not easily be referred to close botanical relation may also occur in some patients. The reactivity to birch pollen, hazel nut and apple is a classic example (19) explained by structural similarities between the major birch pollen allergen, Bet v 1, and a similar protein present in the apple (20). Other plant food proteins are also common sensitizers and testing with isolated or recombinant allergens, like Bet v 1, profilins, lipid transfer proteins (LTP), etc., provides additional information leading to the initial sensitizer in complex cases of multisensitivity and explaining geographical differences in sensitization patterns (21).

Cereals

Cereal grains are often involved in problems of the skin and the gastrointestinal tract. Measurement of IgE antibodies can be especially helpful in cases with a late onset of allergic symptoms (22). Wheat allergens cross-react with grain allergens from rye, barley and oats, and to a minor extent with grass pollen allergens. Testing with allergens of soluble (albumin, globulin) and insoluble (prolamin, glutelin) fractions should be considered (23).

Legume seeds

Sensitivity to peanut is surprisingly common in small children even without known exposure. IgE antibodies against peanut do not decrease as children grow older and sensitivity seems to remain for life (24). Common thickening and stabilizing agents in industrially pre-

pared food contain related proteins from other members of the legume family (25), and may also provide a continuing source of low-dose exposure.

Hidden exposure to legume seeds (peanut, soy, pea) accounts for almost half of the unexpected allergic reactions to food reported in recent surveys in Sweden (26). Several fatal anaphylactic reactions to soy were reported lately. The typical case is a school age child with known allergic asthma to peanut, high anti-peanut IgE and low to moderate anti-soy IgE. The severe reaction may occur more than an hour after consumption of, for example, a meat product containing soy protein. Exercise or low temperature are often involved (27).

Latex

A range of IgE-binding proteins of natural rubber latex and related food products are under study (28). Allergens of some fruits carry epitopes homologous to latex allergens but are most probably different proteins with partial sequence homologies, rather than a universal fruit allergen (29). Compiled data on specific IgE antibody measurement (Pharmacia CAP System) from 11 published studies show agreement with clinical diagnosis in 483/524 cases (92.2%) (30). IgE antibody testing should always be considered in the investigation of suspected latex allergy, in food allergy in children, and in urticaria and anaphylaxis of unknown cause.

Egg, milk and meat

Although the importance of egg white as an allergen decreases with increasing age, some individuals retain their sensitivity to egg as adults. Studies on patients failing to develop tolerance showed that exposure to cage birds can be one reason (31). Such patients usually have IgE antibodies and clinical sensitivity to egg yolk proteins and chicken meat rather than to egg white components.

Cow's milk allergy is important, with a prevalence of about 9% in atopic children (32). Many children showed sensitivity to meat from more than one animal species due to the structural similarities between serum albumins of the animals.

Seafood

Fish may from an allergy point of view be divided into several groups: sharks (dogfish), the order Gadiformes (cod, hake), the Scombroid fishes (tuna, mackerel), and the order Pleuronectiformes (flatfishes). The overlap of allergen specificity between the types varies from moderate to small (33–36). Furthermore, the order Clupeiformes (herrings and sardines) (37), and the family Xiphiidae (swordfish) (38) may constitute allergenically distinct groups. IgE antibodies against *Anisakis* have been demonstrated in patients with

urticaria and gastrointestinal reactions (39). The parasite is common and IgE antibodies has diagnostic importance in reactions to seafood. Presence of cross-reacting allergens to other helminths can not be excluded. However, patients with suspected reactions to fish (40, 41) showed low or no cross-reactivity to *Ascaris* and *Toxocara* with the Pharmacia CAP System.

Crustacean allergens show considerable immunological overlap with insect-derived proteins. Tropomyosin of shrimp also exhibits 80% peptide-sequence identity with mite tropomyosins (42) but plays a very minor role in mite sensitivity (43), probably due to a very low frequency of sensitization to mite tropomyosin.

Interpretation of test results

Measured with a properly designed and standardized assay, detectable concentrations of specific IgE anti-

bodies are not found in non-atopic individuals. The sensitization and development of disease in atopic individuals is a function of allergen exposure. IgE antibodies indicate sensitization and are related to severity of disease but may be found also in asymptomatic individuals who have shown symptoms earlier or who may develop such in the future. Antibodies to one allergen may constitute a major part of the total IgE in monosensitized patients or in the early phase of the development of severe disease (44).

Allergy to one allergen, or even one epitope, may also, through a chemical and immunological relationship to epitopes of proteins from other biological sources, result in clinical allergy to multiple foods and/or inhalants. A rapidly increasing understanding of the role of common sensitizers is needed and can be achieved by application of intelligently selected pure allergen components in carefully standardized immunoassays for specific IgE antibody.

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