

Clinical value and measurement of specific IgE

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Abstract

The diagnosis of human allergic diseases involves the combined use of a careful clinical history, physical examination, and *in vitro* and *in vivo* assay methods for the detection of IgE antibodies of defined allergen specificities. *In vivo* (skin testing) and *in vitro* (measurement of specific IgE in serum) techniques cannot be considered interchangeable, the former reflecting not only the presence of IgE but also mast cell integrity, vascular and neural responsiveness. Both techniques have similarities and differences, advantages and disadvantages. Recently introduced “second generation” immunoassays have continued to improve the analytical sensitivity and reproducibility thanks to automation and improved reagent quality. Quantitative assays may allow the use of specific clinical thresholds able to differentiate symptomatic from asymptomatic patients. False-negative and false-positive results should derive from lability of some major extracts, and from possible cross-reactivities, respectively. Characterization of allergens at a molecular and submolecular level and, where necessary, the use of recombinant allergens can reduce cross-reactions and further improve the quality of immunoassays. © 2003 The Canadian Society of Clinical Chemists. All rights reserved.

Keywords: Allergy; Skin testing; Quantitative assays; Allergen extracts; Recombinant allergens; Standardization; Quality assurance

1. Introduction

Allergic diseases, chronic inflammatory conditions caused by exposure to a variety of external agents, most frequently present as rhinitis, asthma, atopic dermatitis and other skin manifestations, and occasionally as life-threatening anaphylactic shock. Research over the last two decades has led to a better understanding of the causes of, and mechanisms underlying, allergic diseases. Of particular importance was the recognition that a well-defined type of inflammation underlies all allergic diseases, involving a complex interaction between different effector cells and their mediators. Another milestone in this field of research was the identification of a new isotype of human immunoglobulin, called immunoglobulin E (IgE). The medical laboratory has a clear role to play in the detection of allergic diathesis, as well as in the diagnosis and management of patients with allergic diseases. Old and new markers for evaluating eosinophil activation (*e.g.*, eosinophil cationic protein), basophil degranulation (histamine and N-methyl histamine) and mast cell activation (tryptase) are becoming available in clinical laboratories [1]. However, the most diffuse, but

still controversial, type of testing is measurement of specific IgEs in serum. The aim of this paper is therefore to review the advantages and limitations of current assays for specific IgE, and the value of its measurement in clinical practice.

2. Epidemiology: prevalence of allergic diseases

Atopic allergic diseases affect 15 to 25% of the general population [2,3]. There is a growing awareness that the prevalence of atopy and asthma are on the increase in all developed countries. In the USA, where a 75% increase in prevalence since 1980 has been reported, the numbers of Americans with atopic disorders are estimated at: asthma (14.6 million), allergic rhinitis (27 million), atopic dermatitis (10% of children), anaphylaxis (27,500 episodes yearly), food allergy (13.8 million, 5% of children and 1.5% of adults), and venom allergy (1.1–2.8 million) [4]. A recent study designed to objectively assess trends in the frequency of atopy by measuring specific IgE for the eight most common inhalant allergens, demonstrated that between 1987 and 1998 the frequency of atopy in adult Greenlanders increased by almost 90%, equivalent to an 8% increase per year. The increase was greater in 15 to 19 yr olds, but also occurred in older people, thus suggesting that the risk fac-

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tors responsible for the increase in atopy do not operate only in childhood [5]. Despite large variations in different areas (*e.g.*, current asthma prevalence ranges from 2.3% in Finnish children) to consistently above 10% in British children, a large body of evidence demonstrates that the prevalence and frequency of atopic diseases has increased world-wide over the last few decades [6–8]. The reasons for this are only partly understood, but may be related to changes in lifestyle in the western world, including intestinal bacterial flora changes, the use of antibiotics, environmental pollution, obesity and physical inactivity [9]. The role of infections is more controversial: they may exacerbate asthma, but it has been suggested that the control of infectious diseases and the increase in general hygiene in the past decades may have increased atopy. Evidence presented suggests that improvement in sanitation in industrialized countries has reduced the number or type of infections that would have modulated immune development from a TH2 (atopic phenotype) to a TH1 (nonatopic phenotype) [10]. Cost-of-illness studies on asthma and allergic rhinitis suggest that these conditions incur a large burden to society, worldwide. In the USA, healthcare expenditures and direct healthcare costs for these illnesses are calculated at more than 8 billion US dollars a year [11], which is probably an underestimation because most people do not seek healthcare, many using over-the-counter pharmacotherapy. Indirect costs include loss of work productivity, activity limitation, school absences and effects on scholastic carrier and other intangible costs [12].

Difficulties in diagnosing atopic allergy arise from the evidences that several other disease aetiologies are present with similar symptom profiles, and there is no single gold standard for achieving a reliable diagnosis [13], which can only be made by the expert physician when all clinical and laboratory findings have been evaluated. The clinical diagnosis should be based on a careful evaluation of patient's history, symptoms, findings at physical examination, laboratory test results, and any other investigations considered necessary.

A unifying thread linking atopic conditions (*e.g.*, atopic dermatitis, asthma, and allergic rhinitis) is their occurrence in individuals with markedly elevated levels of IgE antibodies [14]. Therefore, from a clinical perspective, the reliable and accurate assessment of specific IgE antibodies is crucial to the selection of patients with atopic allergy and the identification of causative allergens; combined with the clinical history and physical examinations, determinations of specific IgE antibodies are used to establish the diagnosis of allergic disease and to make treatment decisions.

3. Structure and regulation of immunoglobulin E synthesis

IgE was the last Ig class to be discovered because it is a homocytotropic molecule present in the serum in nanogram

amounts, in an equilibrium with that bound to mast cells, basophils, and other cells. This class was easy to demonstrate by skin testing, but difficult to isolate from serum. The discovery, in 1967 by Stig Gunnar Johansson, of a myeloma protein in the serum of a Swedish farmer, initially called IgND, made it possible to prepare rabbit antisera for use in immunoassay [15]. Confirmation also in 1967 that IgND was identical to γ E discovered by Ishizaka and Ishizaka [16,17], and that both were elusive reagents of allergy, prompted further efforts to improving the understanding of the structure and properties of IgE, regulation of its synthesis and its role in allergic diseases. Structurally, human IgE molecules contain two light chains (kappa or lambda) that are indistinguishable from the light chains of the other human immunoglobulins (IgG, IgM, IgA, and IgD). Vice-versa, the two heavy epsilon chains contain five structural domains (VH, Ce1, Ce2, Ce3 and Ce4), that possess unique antigenic attributes and confer its special biologic properties. IgE has a molecular weight of 190,000 daltons, is rich in carbohydrate (12%), and is the only immunoglobulin isotype to contain inter-heavy chain disulfide linkages on either side of the C_H2 domain. This structure may be important for the unique biologic properties of IgE. Serum IgE levels are the lowest concentration of any of the five human immunoglobulin isotypes (0.0005% of total adult serum immunoglobulin levels in nonatopic individuals) and in adults usually are < 200 IU/mL (< 480 μ g/L). Approximately 50% of IgE is distributed in the extravascular space. It has a short biologic half-life of one to five days in peripheral blood which is essentially due to its relatively high fractional catabolic rate (71% intravascular pool catabolized per day). IgE neither passes through the placenta nor activates the classical complement pathway. Its reagenic (mast cell sensitizing) activity depends on its ability to bind to the alpha chain of the high affinity IgE Fc-epsilon receptor (Fc ϵ RI), which resides on the membrane surface of basophils and tissue mast cells [18].

Allergic sensitization is a multi-step, highly regulated process involving both B and T cells whereby presentation of environmental antigens (allergens) to the immune system results in the preferential activation of the TH2 subtype of the CD4+ T-helper lymphocytes population [19]. TH2 cells are programmed to express some cytokines (*i.e.*, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13) upon activation that, in turn, favor humoral immunoresponses, including isotype switching, and IgE production, as well as the other cardinal features of TH2-dependent disorders, first and foremost eosinophilia and goblet cell hyperplasia. All individuals in the general population are exposed to allergens, but only some mount a TH2-dependent IgE response to them.

The specific induction of IgE expression in B cells requires three separate signals. First, engagement of the B cell antigen receptor initiates processes resulting in the activation of a cytoplasmic and nuclear signaling cascade. For B cell activation to proceed to IgE expression, two additional signals are required. The first of these signals is delivered by

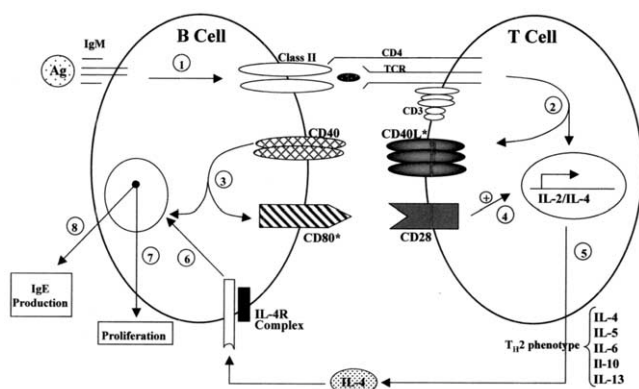


Fig. 1. T-cell interactions involved in IgE synthesis (from reference 21, modified). Binding of an antigen (Ag) by a B cell with surface IgM specific for the Ag leads to [1] internalization of the IgM/Ag complex and is followed by endosomal processing and presentation of the surface of the B cell in the context of HLA class II molecule [2]. Recognition of this complex by an Ag-specific T-cell receptor complex induces CD40L expression [3]. Ligation of CD40 by CD40L results in upregulation of CD80 (B7) expression [4]. Engagement of CD28 by CD80 delivers a costimulatory signal to the T cell which, together with the TCR signal [2], leads to [5] cytokine synthesis and T cell proliferation [6]. The cytokine IL-4 binds to its receptor on B cells and synergizes with the CD40 signal (3 and 6) to induce [7] B-cell proliferation and [8] IgE isotype switching and synthesis.

specific cytokines, and the second results from specific molecular interactions that occur consequent to B cell cognate interaction with activated T cells. The initial cytokine-dependent signal leads to the activation of transcription at a specific region of the immunoglobulin locus, thus determining isotype specificity. The second signal activates recombination mechanisms/machinery, resulting in DNA switch recombination and, in particular, the germline transcription at the ϵ locus, and CD40/CD40 ligand (CD40L/CD154) interactions to trigger recombination [20,21]. The two signals are delivered to B cells by T cells through a complex series of interactions (Figure 1).

Although the basic tenets of this model remain unchallenged, more recent studies from lines of inquiry as far apart as epidemiology and molecular immunology have led to the emergence of novel, more complex paradigms. On the one hand, gene-environment interactions are looming larger and larger as major determinants of the type and amplitude of allergen-induced antibody responses. On the other, the intimate mechanistic connection between transcription and switch recombination has been highlighted by the discovery that switch recombination requires activation-induced cytidine deaminase (AID), a novel RNA editing enzyme. In particular, the so-called “hygiene hypothesis” has focused the attention of allergy researchers on the role that exposure to bacterial products, such as endotoxin or lipopolysaccharide, may have in influencing allergic sensitization. The scenario that emerges from these new insights sees lipopolysaccharide, CD14 and T helper differentiation as players in a complex circuit triggered by different combinations of environmental stimuli, and that can either upregulate or suppress IgE-dependent reactions [22].

Development of the IgE antibody-mediated allergic state involves a sensitization phase and subsequent challenge. In the sensitization phase, activated plasma cells produce IgE antibodies, which bind to the IgE Fc receptors on the surface of basophils in the blood and/or mast cells in the connective tissue of the skin, respiratory and digestive tracts, and around the vessels. When sensitized individuals have second exposure to an allergen (challenge), IgE antibodies cross-link, causing degranulation and the release of multiple chemical mediators (including: histamine, leukotrienes, neutrophil chemotactic factor of anaphylaxis, platelet-activating factor, kinin-producing enzymes—kallikreins; and, in the case of mast cells, prostaglandin D₂). These chemical mediators induce dilation of small blood vessels, increase vascular permeability and postcapillary leakage, constrict smooth muscle in pulmonary blood vessels, increase mucous gland secretion, activate blood platelets, and induce other postinflammatory activities. These events may ultimately lead to an immediate type I hypersensitivity response, ranging from localized cutaneous swelling and erythema (wheal and flare) to more systemic bronchoconstriction, urticaria, angioedema and, in severe cases, anaphylaxis and death [23].

4. *In vivo* measurement of specific IgE

Since the discovery of IgE more than 30 yr ago, controversy remains regarding the advantages and limitations of *in vivo* and *in vitro* techniques for IgE measurement, with studies demonstrating both agreement and disagreement between these techniques [24,25]. In clinical practice, prick (epicutaneous or percutaneous) and intradermal (intracutaneous) skin testing are the most widely used methods for demonstrating IgE antibody activity. Skin testing by the percutaneous route is performed using a diluted allergen that is pricked or scratched into the skin surface. Positive-control skin test (histamine) and negative-control skin test (diluent) are essential for correct interpretation of skin test reactions. About 15 min after the application of allergen to skin, the test site is examined for a wheal and flare reaction. A positive skin test reaction (typically, a wheal 3 mm greater in diameter than the negative control reaction, accompanied by surrounding erythema) reflects the presence of mast cell-bound IgE specific to the tested allergen [26]. Intradermal testing is performed by injecting allergens within the dermal layer and it is usually reserved for venom and drug allergy testing when percutaneous tests are negative but there is high suspicion of allergy. The test allergen is 10- to 100-fold more diluted and although this approach may increase the clinical sensitivity of skin testing, it does so with a loss of clinical specificity. Results of both percutaneous and intradermal testing depend not only on the presence of IgE antibody, but also on mast cell integrity, and vascular and neural responsiveness. Since the latter two factors are relatively constant the skin test is believed to largely reflect

the presence or absence of specific IgE antibodies but, from a theoretical viewpoint, *in vivo* and *in vitro* of measurements are not interchangeable. Many variables have been reported to affect the response to allergy skin testing, including season, time of day, and area of the body tested. In epidemiologic studies there is convincing evidence that the reaction of the subject's skin to histamine is a strong contributor to the results of skin testing [27]. A greater than twofold variation in up to 34% of patients with duplicate skin tests has been reported [28], and there is a marked variation in response with both different allergens [29] and different batches of allergen from the same manufacturers [30]. Another factor that should affect the results of skin is the use of medications, in particular drugs with antihistamine properties, including a number of antidepressants. Antihistamines interfere with the development of the wheal and flare reaction and should be stopped before immediate-type skin testing. First-generation antihistamines may be stopped two or three days before testing, but the newer, second-generation antihistamines can affect skin testing results for three to ten days or longer. Medications with antihistamine properties, such as anticholinergic agents, phenothiazine, and tricyclic antidepressant, also should be discontinued before skin testing. Histamine H₂-receptor antagonists (*e.g.*, cimetidine and ranitidine) have a limited inhibitory effect and may be stopped on the day of skin testing. Inhaled and short-term systemic corticosteroids generally do not significantly suppress the wheal and flare reaction of immediate-type skin tests [31]. The most commonly cited advantages of skin testing are that they are inexpensive and immediately accessible. An added advantage not commonly achieved with most testing today is that the results are visible to a doubting patient. Moreover, clinical reasoning is facilitated, the physician being able to provide the patient with an overall evaluation of clinical history, symptoms, and test results together, in the face of the patient.

5. *In vitro* measurement of specific IgE

In vitro allergy tests detect allergen-specific IgE in a patient's blood using commercially available immunoassays. Differences in design and properties of available immunoassays will be discussed later in the paper. *In vitro* tests for IgE antibody have potential advantages not readily available for skin tests including reliable definition of the performance parameters (sensitivity, specificity, precision, stability, *etc.*). Solid-phase immunoassay reagents have long shelf lives and are amenable to ongoing quality control for stability and reproducibility, which is not easily accomplished in an office with skin tests. As the results of *in vitro* tests are not influenced by pharmacotherapy, medications do not have to be withdrawn before testing; the risk of systemic reactions can also be avoided [25]. For these reasons, *in vitro* testing is the preferred method for IgE measurement in the following cases: a) extreme sensitivity to

allergens, as demonstrated by a near-fatal reaction to an allergen; b) abnormal skin conditions, such as severe dermatographism and active dermatitis; c) during the refractory period of mast cell mediator depletion immediately after a severe allergic reaction; d) use of medications, as already mentioned, that can interfere with skin reactivity; e) uncooperative patients or patients who eventually refuse skin testing [32]. According to the position statement of the Executive Committee of the American Academy of Allergy and Immunology, "selective use of *in vitro* tests can be justified in patients with extensive dermatitis or dermatographism, in patients who cannot be withdrawn from medications that interfere with proper skin testing, and occasionally, in patients who otherwise refuse skin testing" [33].

6. Skin testing vs. immunoassay for specific IgE

In the ideal circumstance of having a purified venom source for both titrated intradermal skin testing and preparation of an immunoassay solid phase, Ford *et al.* reported 100% sensitivity and 100% specificity when a background-based cutoff was used in the immunoassay [34]. The different sensitivity and specificity values obtained in other studies partly depend on differences in inclusion criteria and the reference standard used for classifying patients, and also in part on the quality of the assay used [35–37]. In studies in which the skin prick test was accepted as the gold standard, *in vitro* testing has proved less sensitive [38]. When the reference standard was based on both clinical history and skin prick test, *in vitro* assays and particularly "second generation" assays, showed an excellent clinical efficiency (98%) and specificity (100%) [39]. We found a close agreement between skin and *in vitro* testing even if the laboratory test was found to be more accurate, when in cases of discordance the clinical findings confirmed by further sophisticated research laboratory techniques (RAST-inhibition or immunoblot) were considered the reference standard [40]. In the real world of clinical practice, results of skin testing and modern immunoassays are concordant more often than not [24].

No systematic appraisal has been made of the economic issues involved, but reimbursement data indicate that *in vitro* IgE testing is more costly than skin testing, although the latter often seems to be considered part of specialized visit; its cost is therefore partly included in the overall cost of the examination itself. At the simplest level, a comparison of the charge per test demonstrated that costs of *in vitro* assays are approximately 3 to 4 times greater than the per unit charge for skin testing. Considering the actual practice, and in particular the median number of *in vitro* [24] and *in vivo* [50] tests received by Medicare patients in 1996, the difference between patients receiving skin tests vs. *in vitro* tests is smaller and approximately \$65 [41]. Moreover the AAOA (American Academy of Otolaryngic Allergy) determined that is possible to decrease the number of *in vitro*

Table 1

Similarities and differences between skin testing and *in vitro* testing (from reference 25, modified)

Skin testing	In vitro testing
Reflects not only the presence but also biological activity of IgE	Detects the presence of IgE in serum
Multiple methods, devices with different performance characteristics	Standardization of methods in progress
Extract quality can degrade over time, particularly for certain allergens	Solid-phase coupling appears to prevent loss of allergen recognition
Percutaneous and intradermal tests do not present the same clinical sensitivity and specificity	Clinical relevance of low-range positive results under evaluation
Manual technique that requires training skill, and expertise	Semi or totally-automated assays
Various systems used to report results. Results from one allergy practice not interchangeable with results from another allergy practice	Quantitative results for “second generation” assays
No standards set for testing, personnel, quality control, accreditation	Complex federal standards defined by clinical laboratory Improvement Act (CLIA '88) in the U.S. or other accreditation standards
Results available in a few minutes	Results available in several hours to several days
Smaller cost per test	Higher cost per test; smaller difference in total diagnostic charge
Some medications interfere	No interference from medications
Interpretation of test results requires appropriate clinical training and experience	Interpretation of test results in the clinical context requires appropriate knowledge
Primarily a tool for specialists	Can be ordered by any clinician
Some patients may refuse the test	Diagnostic procedure well accepted by all patients
Risk (small) of systemic reaction to testing	Non-invasive test, no risk of sytemic reaction

tests without compromising clinical practice by: a) using screens to remove nonallergic patients from further unnecessary testing; b) eliminating cross-reactive or unusual allergens from a typical panel unless indicated by clinical history [42].

Table 1 illustrates similarities and main differences between *in vivo* and *in vitro* techniques for specific IgE antibody detection.

Figure 2 shows a proposed clinical pathway for the diagnosis and treatment of allergy considering both *in vivo* and *in vitro* testing. In cases where the patient presents with unequivocal symptoms and clinical history, and just one or few allergens are supposed to be the causative agent(s), a selected measurement of specific IgE in blood is suggested. In cases where diagnosis of allergy remains doubtful, or many allergens are suspected to play a causative role, skin testing or a multiallergen screening test in blood should be performed. If positive, a selected measurement of *in vitro* specific IgE with quantitative assays is then requested to properly diagnose and treat the patient.

7. Different assays for *in vitro* measurement of specific IgE antibodies

Over the past decades, the importance and quality of *in vitro* testing for the diagnosis of type I allergies has gradually increased. The prototype for the *in vitro* analysis of IgE antibody in serum was the radioallergosorbent test (RAST), initially described by Wide and colleagues in 1967 [43]. In its original form, the RAST employed a paper disc solid phase to which allergen was covalently attached (allergosorbent) to bind allergen-specific antibodies of all iso-

types (primarily IgE, IgG, IgA) from serum. Following a buffer wash to remove unbound serum proteins, bound IgE was detected with I^{125} -labeled polyclonal antihuman IgE. Results were reported in classes or in arbitrary units by interpolation from a heterologous IgE antibody pollen reference curve. In its original form, the assay included a reference consisting of serial dilutions of a serum sample containing IgE antibodies to birch pollen. The dilutions were selected to construct a calibration curve giving results in arbitrary units (PRU/mL) and internally calibrated against the World Health Organization (WHO) standard. The original test, the so-called Phadebas-RAST, was first made commercially available in 1974 by Pharmacia Laboratories (Uppsala, Sweden), and the trademark “RAST” came to be used, and is still used, colloquially to describe all immunoassays for specific IgE.

Many attempts have been made to improve the original assay: alternative performances devised by Nalebuff and coworkers in 1979 used longer incubation times and a different scoring system to achieve a sensitivity similar to that skin testing, but resulted in loss of specificity [44]. Investigators then tried to replace the original insoluble dextran as a solid phase with other support media, such as cellulose in the form of filter paper discs [45], microcrystalline particles [46], thread [47], minicolumns [48] and paramagnetic particles [49]. Other support media used include nitrocellulose [50], polystyrene tubes [51] and microtiter plates [52]. Many of these approaches, now commercially available, were summarized in a review [1]. More recently, immunoassays have undergone several refinements, including substitution of the original radiolabeled polyclonal antibodies with enzyme-tagged monoclonal detection antibodies, the development of solid-phase support

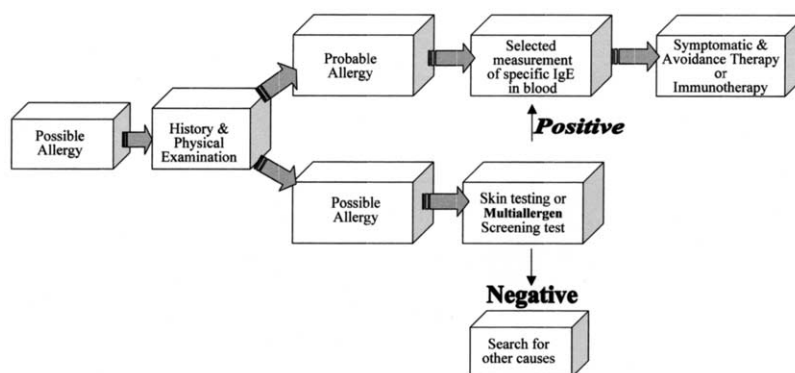


Fig. 2. Clinical Pathway for diagnosis and treatment of allergic diseases.

systems with higher allergen binding capacities, and the introduction of semi-automated systems. Figure 3 shows the two most representative classes of immunoassays for the measurement of specific IgE in blood that are based on solid or liquid phase allergens, respectively. In fact,

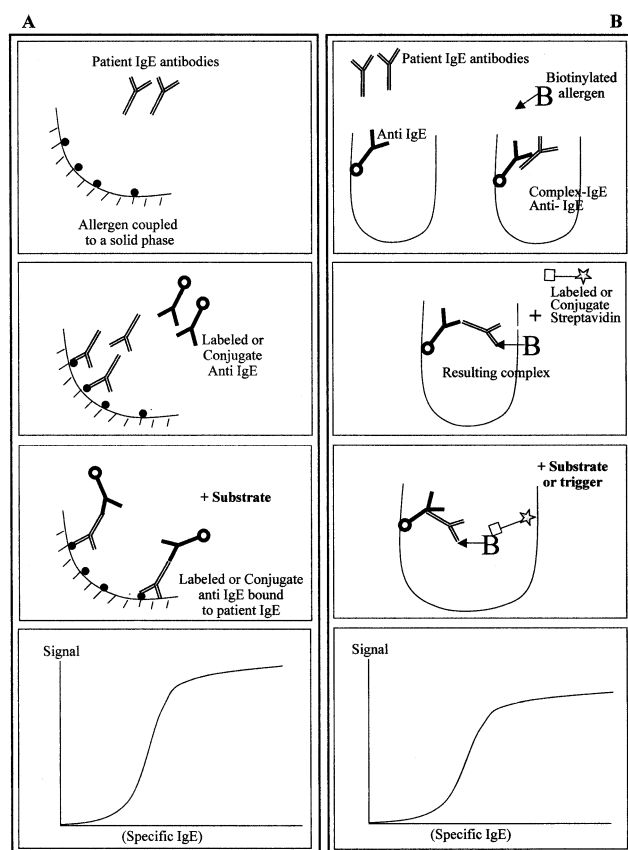


Fig. 3. Main classes of immunoassays for specific IgE measurement. A) immunoassay based on allergens bound to a solid phase. If the tracer is ^{125} I this appears to be a two-site immunoradiometric assay (IRMA). Alternatively, anti-IgE antibodies may be labeled with enzymes or other nonisotopic tracers. B) Sandwich immunoassay based on liquid phase allergens. Streptavidin may be conjugated with peroxidase, acridinium esters or other tracers. The reaction is triggered by adding a specific substrate or other chemicals.

today available immunoassays are noncompetitive assays which employ different tracers, mainly nonisotopic labels, and the main difference is related to the use of solid or liquid phase allergens.

Although, by the early 1980s, the market for *in vitro* allergy testing had grown sufficiently to prompt different companies to develop alternative immunoassays using novel technologies, most physicians are under the impression that the new analytical and clinical performances are the same as those achieved using the original RAST. Therefore, it is time to better characterize and classify immunoassays for specific IgE on the basis of their true performances.

According to NCCLS approved guidelines [53], IgE antibody immunoassays can be classified into qualitative, semiquantitative, or quantitative assay depending on the degree with which the assay result accurately reflects the quantity of IgE antibody in the test specimen, and the assay's precision requirements.

- **Qualitative.** A qualitative (screening) assay produces one of two possible results: either the analyte is 1) nonreactive, negative, nondetectable; or 2) present, or reactive, positive, detectable, or present. These assays generally have a single reference specimen, which defines the positive threshold level of the assay itself. The most diffuse and typical illustration of a qualitative assay is the multiallergen screening test (e.g., Pharmacia Phadiatop) in which five or more different allergen specificities are present in a single reagent and the presence of IgE antibodies to these allergen specificities is evaluated in a single analytical measurement [54–59]. Due to the large number of allergenic specificities, reagents in these assays are more difficult to quality control for the reproducibility of the allergen composition and quantity.
- **Semiquantitative.** The variations in the positive signal are usually presented in a series of increasing grades or classes (e.g., 0 to VI; low to high); in arbitrarily defined units per milliliter (U/mL), or liter (kU/L) determined relative to a supplier-specific, heterolo-

Table 2
Classification of different assays for allergen-specific IgE in vitro (from 53, modified)

Classification	Presentation of Results	Calibration of Methods	References, calibrators and controls
Qualitative	-Positive or negative	-Single or dual calibrators to normalize assay	-One reference sample
Semiquantitative	- indeterminant or “grey” zone -Arbitrary units or classes	-Single or dual calibrators to normalize assay	-Negative and positive control -Negative and bilevel controls (occasionally trilevel controls) -References unique for the test system made from pooled patient sera
Quantitative	-Quantitative Units (kU_A/L) related to a generally recognized reference preparation (e.g., WHO IgE standard 75/702)	-Multipoint standard curve used for interpolation	-Trilevel positive controls -References calibrated towards a qualified reference preparation

gous dose–response curve. The results produced are not traceable to any common reference material, and the assays do not always (consistently) meet the criteria of linearity, dilution recovery, and parallelism that are typically achieved by quantitative assays. The original RAST and most “RAST analogues” should be grouped in this category.

- **Quantitative.** A quantitative assay provides an accurate and reproducible estimate of the concentration of an analyte, such as an IgE antibody, and fulfills the analytical criteria for quantitation, including parallelism, recovery, precision, and linearity across the whole assay’s working range. This type of assay employs a multipoint standard or calibration curve from which unknown data are interpolated. The reference curve can be constructed using heterologous or homologous interpolation methods. The calibrated titration assay that uses heterologous interpolation is unanimously considered an acceptable alternative because IgE antibody for each allergen is interpolated from a heterologous or different IgE reference curve, such as a total serum IgE reference curve [53]. Therefore the assay has to be traceable to the WHO 75/502, internationally recognized, total serum IgE standard. The results can be reported in gravimetric (mg/L) and/or international units (kU_A/L).

Table 2 summarizes the main characteristics of the three different classes of methods.

While qualitative assays maintain their value in clinical practice, particularly when used as a first step in the diagnostic algorithm and/or as an objective tool in epidemiologic studies, semiquantitative assays have several limitations. In the position statement of the American Academy of Allergy and Immunology [33], the accurate quantitation of IgE measurement is considered “a necessary prerequisite to defining more precisely the diagnostic accuracy of IgE *in vitro* tests and the relationship between quantity of IgE antibody and the symptoms or risks of disease”. Moreover, it facilitates the usual statistical criteria for threshold selection (e.g., ROC analysis) and determination of precision

profiles [60,61]. Pastorello and colleagues well demonstrated that threshold levels for specific IgE are likely to be useful in clinical practice to distinguish symptomatic from asymptomatic allergy in patients with positive skin test results [62]. This finding should overcome a possible limitation of IgE measurement, which is the evidence that detection of IgE antibodies may merely reflect the existence of sensitization to given allergens and that their presence does not always coincide with a clinically significant allergic disease. Therefore, quantitative results might really have an added value in clinical practice, allowing physicians not only to detect the presence of sensitization to a given allergen, but also to relate IgE concentrations to symptoms and risk of diseases. Furthermore, the arbitrary reference system with myriad class-scoring schemes should be abandoned in favor of quantitative reporting methods in which test results are reported in units proportional to antibody content, thus favoring clinical reasoning and avoiding confusion.

In theory, all “second-generation” techniques for specific IgE measurement, which are calibrated against the WHO Standard 75/502, should be considered “quantitative” assays. However, Williams and colleagues evaluated commercially available assays for the measurement of IgE antibodies against analytic performance criteria and, in particular, a slope of unit over the useful measurement range for all allergens, low variation across replicate measurements, statistically determined limits of detection, and defined saturation points. Four out five assays demonstrated substandard overall performance with multiple instances of poor precision and accuracy, particularly for certain allergens, such as weeds and molds [63]. In another paper from our group, significant discrepancies were detected on comparing four “second generation” assays, although most clinical discrepancies were reduced by selecting method-specific thresholds using ROC (receiver-operating characteristics) analysis [64]. An analysis of these data suggests that second-generation techniques standardized against the 75/502 WHO standard only partly improved intermethod agreement and clinical discrepancies. Data from different assay systems and laboratories are not yet interchangeable. The assay

architecture and the quality of allergen extracts, as well as the quality of other reagents, significantly affect analytical performances and may cause eventual deviations from an ideal assay.

7.1. Qualification of IgE antibody assays

The analytical performances of immunoassays for IgE antibody measurement depend on the whole architecture of the method, but particularly on some fundamental variables. Each variable plays an independent role but may also affect other variables, thus having additional effects on the final quality of the assay. The fundamental variables of immunoassays for IgE antibody are as follows:

7.1.1. Antihuman IgE antibodies

Antibodies specific for human IgE are used as both capture and detection reagents. Once purified, the antihuman IgE reagent antibodies are either used directly as a solution phase antibody or subjected to chemical modifications in the form of radio-labeling, enzyme-labeling or chemical and physical immobilization on solid-phase matrices. As these key-reagents confer specificity on assays, they must be highly specific for unique determinants on epsilon heavy chains [65,66]. Initially, polyclonal reagents were prepared by immunizing animals (*e.g.*, rabbit, goat, sheep, horse) with purified IgE myeloma proteins [67,68]. More recently, monoclonal antibodies specific for human IgE have been made available, and are used both for research and commercial immunoassays [69,70]. Monoclonal reagents are specific and lack batch-to-batch variations usually observed for polyclonal antisera [71]. However, as they have low affinity and avidity, some manufacturers employ mixtures of monoclonal and polyclonal antihuman IgE as capture or detection reagents. The specificity of human IgE reagents can be documented both by direct binding (dilutional analysis) using insolubilized purified human immunoglobulins and by competitive inhibition, by including non-IgE immunoglobulins in the assay system.

7.1.2. Allergen-containing reagents

The allergen-containing reagent, the critical component conferring allergen specificity on the IgE antibody immunoassay, is unanimously considered the most complex and variable component of immunoassays for IgE measurement in terms of their preparation from raw biologic material and their interbatch quality control and validation. The heterogeneity of this reagent has many possible causes, even when the same manufacturer utilizes the same extract source, chemicals and manufacturing procedures (Table 3).

The importance of quality and standardization of allergy extracts in allergy practice has long been acknowledged, and in 1944, at the first meeting of the American Academy of Allergy, Louis Tuft stated: “I think all of us will agree that standardization of extracts in their present forms is highly desirable” [72]. The quality of natural allergen ex-

Table 3

Potential causes of allergen-containing reagent heterogeneity (from reference 53, modified)

- allergen source: misidentification, contamination and inherent biological variation of the raw material (53)
- extraction process: interlot variation in allergen composition (53);
- allergen-reagent manufacturing: interlot variation (53);
- variable binding of allergen to labels or solid supports (71);
- variable stability of allergen during storage and possible loss of some allergen
- specificities (71);
- heterogeneous internal allergen reference standards used in quality control (53);
- heterogeneous IgE antibody specificities in human serum quality controls materials (53);
- different assays and acceptance criteria used in validation of final reagents (64).

tracts has improved during the past decades as knowledge of the protein chemistry of allergens has grown, and has been applied by manufacturers to produce more consistent products [72–79]. Allergen standardization is no longer based on protein nitrogen units or weight/volume estimates. However, allergens prepared from natural source materials remain heterogeneous products containing many nonallergenic proteins and other macromolecules and, to date, only a few allergen extracts preparations out of the hundreds available, have been approved by the WHO for use as international standards (Table 4).

Moreover, allergens produced from natural source materials vary in allergen composition and content, might be contaminated with allergens from other sources, and can contain proteolytic enzymes [81]. Most allergen sources contain multiple major and minor allergens and, even with the use of modern techniques, it is difficult to standardize a mixture of different proteins. The advent of recombinant allergens offers exciting new perspectives in immunotherapy as well as in diagnostic testing [82,83]. In recent years, there has been tremendous progress in the molecular biology of allergens; all the major allergens have now been cloned and an increasing number of recombinant allergens have become available, representing a significant proportion of the epitope complexity of natural allergen extracts (Table 5).

Table 4

WHO International Biological Reference Preparations for Allergens (80)

Common name	Genus/Species	Approved by WHO in	WHO code
Birch tree	<i>Betula verrucosa</i>	1986	86/522
Common ragweed	<i>Ambrosia artemisiifolia</i>	1983	84/581
Dog hair/dander	<i>Canis familiaris</i>	1986	84/685
House dust mite	<i>Dermatophagoides p.</i>	1983	82/518
Timothy grass	<i>Phleum pratense</i>	1983	82/520
Bermuda grass	<i>Cynodon dactylon</i>	Pending	na
Rye grass	<i>Lolium perenne</i>	Pending	na
Alternaria extract	<i>Alternaria alternata</i>	Pending	na

Table 5
Advantages of recombinant allergens for diagnostic purposes

-
- they can consistently be produced at high purity (82);
 - reagents are specific and more amenable to standardization in mass units (82);
 - they can be produced at defined concentrations with verifiable protein content (83);
 - they can be loaded with great efficiency onto capture supports (83);
 - they provide greater specificity and fewer problems with spurious cross-reactivities than natural allergen extracts (82);
 - for all the main sources of allergens (mites, pollens, animal dander, cockroach and foods) already exist or are under developed recombinant antigens that could be used as a single specificity or in cocktails for diagnostic purposes (83);
 - by careful allergen selection and formulation of the cocktail, the allergenic activity of the natural product could be reproduced with recombinant allergens (83)
-

In addition, it has been shown that spiking of allergen extracts with recombinant allergens that are not well represented in allergen extracts can improve the sensitivity of diagnostic tests [84,85]. Finally, recombinant allergens are essential for the development of innovative techniques using microchip technologies and aiming to improve our understanding of problems related to cross-reactivity of IgE antibodies to allergens [86].

7.1.3. Calibration system

The third component of immunoassays for IgE that varies widely among commercially available assays is the calibration system used to define the level of IgE antibody that is generated by the assay. In semiquantitative assays, the magnitude of the signal is related in terms of rank order to the quantity of IgE antibody present in the test serum, but is not consistently directly proportional to the quantity itself. Quantitative assays employ the most advanced methods of calibration, using both homologous and heterologous interpolation methods. However, because of constraints placed on assays using the homologous interpolation calibration as a result of limited IgE antibody-containing human serum pools, the heterologous interpolation procedure has been considered the most feasible current calibration strategy. In most “second generation” methods a total serum IgE calibration curve is carried out simultaneously with the allergen-specific IgE portion of the assay. This heterologous interpolation scheme uses an IgE calibrator that is traceable to a primary IgE standard, such as the WHO 75/502, and is considered the most generic and practical available solution to the problem of interlaboratory cross-standardization. In some commercially available methods, thanks to automation and reagent stability, assay systems that store a multi-point reference curve in computer memory for a number of days have been developed. Control specimens are then analyzed to validate the reference curve stored in memory for the specific assay run. However, not all currently available assays calibrated against the WHO 75/502 allow the clinical laboratory to obtain accurate and reproducible re-

sults. On using samples in a dilution series and examining the reported results by means of regression analysis, Williams and colleagues found that the average overall slope for most assays, except for the Pharmacia CAP system, did not favorably compare with the ideal standard [63]. These deviations from the ideal reflect the inability of these assays to accurately quantitate different concentrations of specific IgE antibodies. Another relevant observation is the lack of precision in some of current assays in their analysis in triplicates. In fact, all analytical variables and the whole architecture of the assay can significantly influence final results and inter-assay agreement. Recently developed immunoassays by Bayer Diagnostics (ADVIA Centaur, Bayer Diagnostics, Tarrytown, NY, USA), Diagnostic Product Corporation (IMMULITE 2000, DPC, Los Angeles, CA, USA) and Adaltis (ALLERgen, Adaltis Inc., Montreal, Quebec, Canada), that present interesting analytical characteristics, are under evaluation for demonstrating their efficiency and effectiveness in clinical practice.

7.1.4. Solid-phase and other variables

The quality and binding capacity of the solid-phase is critical in assuring total quality in assays for IgE measurement, and this has led to the introduction of a series of novel solid-phase materials with a higher binding capacity. In the Pharmacia Cap system, which can be considered the prototype of “second generation” assays, the new flexible hydrophilic polymer encased in a capsule can bind three times as many proteic allergens compared to conventional cellulose discs [87]. From the Law of Mass Action applied to heterogeneous solid-phase immunoassays, it can be concluded that when the concentration of the allergen multiplied with the equilibrium constant exceeds 10, more than 90% of IgE antibodies are bound, and the reaction becomes antibody affinity independent [13,65]. Therefore, the solid-phase and all allergen components need to be in large excess to provide such a high binding capacity. Immunoblotting experiments have demonstrated that virtually all IgE antibodies are bound in serum samples to the allergens on the ImmunoCAP [88]. Other commercially available assays fulfil such criteria [63,89]. Also the tracer and the signal detection system influence the sensitivity and quality of final results. Immunoassays once used radioiodinated recognition antibodies, whereas modern assays employ enzyme-labeled antibodies and spectrophotometry, fluorometry, or chemiluminescence for signal detection, thus avoiding problems related to the use of isotopic reagents, and allowing automated analytical procedures.

7.2. Quality and clinical value of current assays for IgE antibody

Although most physicians still request the “RAST” and consider the analytical performances of modern assays for IgE antibody comparable to those of “RAST and RAST-analogues” assays, the quality, standardization and impor-

tance of *in vitro* techniques for the diagnosis of type I allergies have gradually increased over the past decades. However, an *in vitro* test response cannot always automatically be translated into a clear clinical diagnosis. Better utilization of *in vitro* testing for IgE antibody calls for improvements in both the analytical quality of current assays and in postanalytical interpretative criteria.

Following a recent conference on aspects of quantitation of IgE antibodies in allergic diseases, Yunginger and other experts reviewed the current role and benefits of quantitative assays for IgE antibody [90]. The first clinical utilization of quantitative IgE is for predicting development of atopic disorders. Although an elevated cord blood total IgE level may be useful in identifying high-risk children, the development of a more specific indicator atopic marker [91], such as a food- or aeroallergen-specific IgE antibody level, is in great demand. Many studies have demonstrated the (positive and negative) predictive value of such a marker for development of atopic diseases, and further trials are underway to evaluate the most appropriate age of children to demonstrate sensitization, and the most effective thresholds [92–96]. The identification of high-risk children by measuring IgE antibodies before manifest and clear-cut clinical allergy symptoms have appeared, the so-called Allergy March [97,98], becomes paramount for the introduction of secondary and tertiary allergy prevention [99–101]. In food allergy, the performance characteristics of quantitative immunoassays assure a high positive predictive value (ranging from 90–95%) and can reduce the need for oral challenges by about 50% [102–105]. Monitoring the concentrations of specific IgE antibody to foods such as milk, egg, soy and wheat holds promise for monitoring dietary avoidance and for predicting when tolerance will develop [106–108]. An increasing body of evidence supports the use of quantitative IgE antibody testing for the common inhalant allergens, and strongly suggests that its measurement is an accurate measure of individual patient sensitivities [109–111]; the adoption of an appropriate clinical threshold may allow the physicians to distinguish between symptomatic and asymptomatic patients [62]. IgE antibody measurement may serve as a marker of current allergen exposure, and as a tool for monitoring allergen avoidance [101–109]. Other clinically important specific uses of quantitative IgE antibody assays are insect venom allergy, thus replacing the skin tests that present an intrinsic risk for patients [112], and latex allergy [113,114].

As for any other laboratory test, there are two possible causes for disagreement of *in vitro* IgE antibody results with clinical findings. First, problems might derive from false-negative test responses that, in turn, are mainly due to lability of some major allergens, particularly important in food extracts. A well-known example of a labile food allergen is the major allergen in apples, Mal d1 [115]. This also applies to homologous allergens in other fruit extracts, such as peaches, cherries and pears [116], which affect both *in vivo* and *in vitro* techniques for IgE measurement. To

overcome this limitation, many allergologists use the so-called prick-by-prick technique for skin testing using a “fresh” allergen, without any purification or preliminary treatment. This may resolve the lack of reactivity by some extract preparations, but it carries additional concerns about reproducibility, triggering systemic reactions, and increased false-positive results [24,25]. Our group recently demonstrated that in patients suffering from gastrointestinal symptoms after wheat ingestion, all the atopic patients, and only one of the nonatopic patients, were positive to both wheat CAP and skin prick test. In the latter group, the positive immunoblotting results contrasted with the responses of both *in vivo* and *in vitro* tests, indicating that currently used allergenic wheat protein preparations are of limited value in detecting specific IgE to wheat [117]. A better characterization of all determinants of allergic reactions to some allergens and, where necessary, the identification of all clinically important epitopes, enhance the feasibility and clinical effectiveness of the routine diagnostic methods for IgE detection and quantitation. Therefore, a better characterization of allergens structure at the molecular (allergen molecules) and submolecular (epitopes) level is a prerequisite for improving the sensitivity and overall quality of IgE testing. The second possible reason for a poor predictive value of *in vitro* IgE results is related to false-positive test responses caused by cross-reactivity.

8. Cross-reactivity and co-sensitization

The ambiguous term, “cross-reactivity”, is sometimes used inappropriately. The frequently observed concomitant positivity to different allergy extracts, mainly due to the widespread use of skin and serologic tests in clinical practice, calls for a basic distinction between cross-reactions and co-sensitivity. Cross-reactions may be limited to the presence of cross-reacting antibodies with different affinities without any clinical expression, cross-reactivity being demonstrated only by *in vitro* techniques under antigen excess conditions [118]. Viceversa, co-sensitization corresponds to allergic symptoms provoked by various offending allergen sources containing common allergen structures at the molecular and submolecular level [119]. The fact that most allergic patients react to more than one allergen largely reflects multiple sensitization events consequent to the increased immunologic responsiveness present in atopy. A TH2 response to one allergen is likely to facilitate subsequent TH2 responses by creating and maintaining a local milieu favoring TH2 responses, and this is known as the “IgE breeds IgE” phenomenon [120]. However, even for allergens that are simultaneously presented to the immune system, co-sensitization is not universal [121]. Another cause of allergic multireactivity is *in vivo* cross-reactivity. The definition of cross-reactivity is based on immunologic recognition: two allergens are cross-reactive if there is a single antibody (or T-cell receptor) that reacts with both.

Table 6

Associative mechanisms resulting in clinical concomitant sensitivity (from Steinman HA (136), modified)

Type of mechanism	Examples	Explanation
1. Similar protein, similar or related species	Rye and Timothy grass	Family relationship (identical allergens)
2. Similar proteins, dissimilar or unrelated species	Olive, privet, ryegrass	Cross-reactivity demonstrated but panallergen not identified
3. Panallergens	Peach, peanut, apple	Presence of identified panallergens/(e.g., profilin)
4. Proximal occurrence caused by house dust mite	Feather pillow allergy	Allergen in the same location (e.g., mites in pillows)
5. Clustering	The most common foods associated with melon allergy (avocado, banana, kiwi, peach)	Statistical event without a demonstrated biochemical or clinical basis

Three points in time and three different cells are involved in cross-reactivity. The first point in time is the immunologic prime and, at this stage, the allergen has to be recognized by virgin T and B cells. The second point in time is the contact of the allergen with memory T and B cells, and the last point in time is the contact between the allergen and the sensitized mast cells [122]. In many cases of symptomatic cross-reactivity between allergens, the immune system has probably been triggered by only one of the cross-reacting partners, which reacts with cell-bound antibodies on the mast cells near the mucosal surface, but does not reach the immune system itself. Different hypotheses have been proposed to explain the relatively high prevalence of cross-reacting antibodies, in particular by three different mechanisms. The first is partially related to the phenomenon called “the original antigenic sin” [123], a metaphor used to describe the effect that the first viral infection appears to have on the immune system as compared to subsequent infections with viral variants. The second mechanism is polyclonality, and the increase in polyclonality following repeated exposure to the same antigen: the more diverse the antibody repertoire, the more likely it is to cause some cross-reactivity in an individual. The third mechanism, affinity maturation, basically consists of an increase in antibody affinity after repeated antigen contact [120]. Some of the highlights in the history of unexpected cross-reactions in allergy are ragweed/banana, first detected in 1970 [124], birch/apple [125], cross-reacting carbohydrate determinants [126,127], profilin [128,129], mugwort/celery [130], latex/banana/avocado [131], and mite/snail/shrimp [132–134]. The birch/apple story started in 1977, when Hannuksela & Lahti described the association on clinical grounds [125]. At that time it was difficult to identify and characterize cross-reactions because extracts were of poor quality, deficient in relevant proteins, and contained cross-reacting structures. Our knowledge of cross-reactivities has improved thanks to the better quality of allergen extracts and the evidence of simultaneous positive cutaneous tests and presence of serum specific IgE to different extracts. It was once impossible to differentiate cross-reactivity from co-sensitization. A significant improvement was made in the midseventies, using the so-called RAST-inhibition with crude extracts. IgE inhibi-

tion by the allergen extract containing the cross-reactive allergen allowed us to establish the presence of cross-reactivity, but it did not permit the identification of the allergens involved. Further advances came with techniques for separating different allergens into crude extracts (immunoblot and cross-radio electrophoresis). Comparative immunoblottings of allergenic fractions of two different allergens gave the molecular weight and/or isoelectric points of potentially cross-reacting fractions. By using a large number of sera from patients allergic to different extracts, it was possible to identify the number of patients showing similar IgE binding patterns. The cross-reactive allergens were usually defined by their physicochemical properties, but their molecular nature was not clarified. Only since the early nineties have purified and recombinant allergens and monoclonal antibodies specific for the purified allergen been used in these investigations, and the use of recombinant allergen in quantified cutaneous tests has allowed the identification of cross-reactive allergens at a molecular level [135]. A classification of associated mechanisms resulting in concomitant clinical sensitivity (CCS) is reported in Table 6.

- **Similar proteins, similar or related species.** In general, cross-reactivity reflects the phylogenetic relation between organisms: the closer the botanical relationship between two plants, the greater the degree of structural and immunologic similarity of the allergens [137]. Structural similarities require homology in primary sequence that results in homologous 3-D structures. Serum albumins from vertebrates are often cross-reactive; homologous allergens from phylogenetically related grasses tend to be cross-reactive, and IgE antibodies to peanuts are often reactive with homologous proteins in soybean and other legumes [138].
- **Similar proteins, dissimilar or unrelated species.** Cross-reactivity between two or more dissimilar taxonomical species has been recognized for over three decades. The “celery-spice-carrot-mugwort syndrome”, an example of this associative mechanism, is due to one or more allergens that appear to be similar but occur in dissimilar species [139].

- **Panallergens.** Sensitization to multiple pollen species is an usual finding and the identification of allergenic molecules with a high level of homology in the pollen of different plant families led to the discovery of “panallergens”, examples of which are profilin, Bet v 1-homologues, chitinases, lipid transfer proteins, tropomyosin, cyclophilins, and lipocalin. Profilins, probably the most diffuse panallergen, were discovered as highly cross-reactive allergens and important components of the plant cytoskeleton by the isolation of a cDNA coding for cross-reactive birch pollen allergen, and its identification as profilin. There is extensive cross-reactivity of allergic patients’ IgE antibodies with profilins from different plant sources but also from other organisms, including man. Profilins, well established as clinically relevant cross-reactive allergens in tree, grass and weed pollens, are also expressed (at lower levels) in somatic plant tissues (e.g., fruits, leaves, seeds and roots) [140–144].
- **Proximal occurrence.** Occurring when an adverse effect is caused not by the apparent allergen but by another allergen proximal or physically associated with it, examples of proximal occurrence are Red Spider Mite on tomato, Baker’s Asthma and hidden allergens in food, cosmetics and medications [145].
- **Clustering.** The clustering of allergens will be specific for certain locations, occupations or groups of people, beyond what is explained by other known CCS mechanisms [146].

From a clinical viewpoint, further knowledge is required on patterns of cross-reactivity because they often reflect the pattern of clinical sensitivities and related allergic diseases. From an immunologic viewpoint, a greater understanding of these phenomena could improve our knowledge of the regulation of allergic sensitization and, finally, from a therapeutic viewpoint, it may be useful to predict further possible allergic reactivity to novel foods and allergens. Other important benefits may derive from a greater knowledge of cross-reactions: for example, great similarity between two allergens does not increase the diagnostic accuracy to include both in a diagnostic panel. Likewise, successful desensitization with one allergen is likely to relieve the symptoms of the other very similar agents. Significant improvements in our knowledge of types, prevalence and molecular determinants of cross-reactions has been achieved in recent decades, and further improvements should derive from the availability of recombinant allergens. Recombinant allergens can be used not only as a tool for research, but also as reagents in modern immunoassays, thus reducing “spurious” and clinically unimportant cross-reactions. Distinguishing between cross-reactivity and co-sensitization calls for *in vitro* experiments [147]. However, ambiguities and complexities must be kept constantly in mind. Cross-reactivity *in vitro* is not always reflected by cross-reactivity *in vivo* and the differentiation between IgE

binding and biologic activity requires a close collaboration between clinicians and laboratory specialists. A recent review on the clinical implications of cross-reactive food allergens demonstrated the low risk of clinical reactivity for families of food antigens that present *in vitro* cross-reactivities to other related food allergens [148]. Factors that determine the clinical appearance of an allergy in the face of sensitization are complex and related to both host (immune response and target-organ hyperreactivity) and allergen (lability and digestibility) and, therefore, a careful clinical appraisal calls for laboratory investigations as well as the knowledge of clinical history, symptoms and frequently oral double-blind, placebo-controlled, oral food challenges or other sophisticated examinations. In practice, if two allergens are very similar, it does not increase the diagnostic accuracy to include both in the diagnostic test panel.

8.1. Quality assurance and quality control

Quality assurance for IgE antibody assays first depends on manufacturers providing specifications for all aspects of production in verifying reagent quality and performance. Recent years have seen an increased regulatory focus on testing systems, and it is the ethical and legal responsibility of *in vitro* IgE test manufacturers to assure the quality of their products [33], and provide clinical laboratories with essential information on reagents used, the source and quality of allergen extracts, data on stability and lot-to-lot consistency, and performance characteristics (reproducibility, sensitivity, specificity, etc.) of the assay offered. However, the individual laboratory maintains the responsibility for the verification and validation of the performance characteristics of these regulated assays.

The second factor in quality assurance is internal quality control (IQC), that must be conducted by the individual clinical laboratory to demonstrate that the assay is “in control”. Individual strategies adopted by the specific laboratory vary depending on the assay design and equipment used, and also on two extreme positions taken. One expresses the view that each allergen-containing reagent is a separate test that should have its own specific control serum, which should be tested in each analytical run. The more achievable extreme is to consider the IgE antibody assay a system employing several hundred different allergen-containing reagent “components”. As such, it is assumed that each reagent has already been quality-controlled by the manufacturer. The most practical proposal is to identify a group of common allergen specificities, to prepare or acquire sera with these specificities and analyze it in each assay by, if necessary, rotating these and other allergen specificities during different analytical runs. The third quality assurance level is participation in external quality assurance (EQA) or Proficiency Testing (PT) schemes. Currently, there are several national and international programs dealing with IgE antibody assays and their primary goal is to identify laboratories and assays that accurately measure

IgE antibodies of different specificities and to allow individual laboratories to compare each others' results. In recent years interlaboratory agreement has significantly improved, particularly in the case of clinical laboratories that adopt "second generation" assays assuring reliable quantitative results [149].

8.2. The future

Immunoassays for the IgE antibody have undergone significant improvements, with the utilization of more purified allergenic extracts, the capacity to quantitate IgE antibodies in mass units, and total automation of assay systems. However, current assays are time-consuming, require large quantities of serum samples, do not employ recombinant allergens or cocktails of recombinant allergens, and the throughput is not adequate for screening for the most common allergens at reasonable costs. Therefore, efforts are in progress to overcome present limitations. High-throughput, nonisotopic systems totally dedicated to allergy testing or consolidated with other clinical chemistry analyses, and using, at least in part, recombinant allergens have been or will soon be introduced in clinical laboratories. Other sophisticated technologies are under development. High-density ordered arrays of molecules (microarrays), for example, may circumvent most of the current limitations by allowing the simultaneous and multiparametric analysis of serum reactivity against a variety of antigens [150]. The ability to multiplex microarrays allows the production of assays that can simultaneously screen hundreds of specificities both of the same allergen and of different related or significant allergens thus improving the knowledge of the molecular and submolecular basis of allergic diseases [151,152]. However, allergy testing is the prototype of the new role that laboratory medicine and laboratory specialists must play in the near future. In fact, improvements in analytical quality are necessary, but not enough to assure an adequate "clinical" quality to laboratory services. Appropriateness in test requesting is a prerequisite for improving the efficacy of clinical laboratories in allergic diseases and, therefore, the generic request of "RAST" without selecting suspected and relevant allergens can no longer be accepted. Some allergens that appear to cross-react with other specificities may prove to be without any clinical relevance, and the inclusion of very similar allergens in the diagnostic panel does not increase the clinical accuracy. The interpretation of quantitative results might be based on well-selected thresholds that allow to graduate the severity of allergic sensitization and, possibly, to differentiate atopy from symptomatic diseases. Laboratory information should be totally integrated in rational clinical pathways with skin testing and other diagnostic modalities, and, finally, laboratory specialists should be viewed by physicians as consultants in a patient-oriented process.

9. Glossary

Allergen: an immunogen that when introduced into an immunocompetent, and predisposed host elicits the formation of IgE antibodies.

Allergen extract: a mixture of glycoproteins, lipoproteins, or protein-conjugate chemicals/drugs that have been solubilized from a defined source, and that can elicit an IgE antibody response in exposed persons.

Allergen source material (raw material): the starting raw material from which allergenic extracts are obtained.

Atopy: the inherited tendency to develop immediate-type hypersensitivity to common, and generally harmless substances.

Binding capacity: number of human IgE antibody molecules that an allergen-containing reagent can bind reproducibly under standardized assay conditions.

Conjugate: an assay reagent that is produced by covalently attaching two or more substances to each other such as an antibody with a second biolabel (enzyme or biotin), radiolabel, or fluorophor.

Co-sensitization: allergic symptoms provoked by various offending allergen sources containing common allergen structures at the molecular, and submolecular level.

Cross-reactivity: the reaction of an antibody with an antigen other than that which elicited its formation, as a result of shared, similar, or identical antigenic determinants.

Epitope: 1) a single antigenic determinant. It is functionally the portion of an antigen that combines with an antibody paratope, 2) the minimum molecular structure of the antigenic site that will react with a monoclonal antibody

Heterologous interpolation: a calibration scheme in which the standard or reference dose-response curve is constructed using reagents that have a different (heterologous) specificity from those being used to measure the analyte of interest. In the context of specific IgE assays, this means that a total serum IgE standard (calibration) curve is used.

Homologous interpolation: a calibration scheme in which the standard or reference (calibration) dose-response curve is constructed using reagents that have the same (homologous) specificity from those being used to measure the analyte of interest (e.g. in an assay that measures IgE anticommon ragweed, the calibration curve is constructed with multiple dilutions of a serum containing a predefined amount of ragweed-specific IgE antibody).

Idiotypic: the antigenic characteristic of the antibody variable region.

Radioallergosorbent test (RAST): the first immunosystem available for the measurement of human IgE antibodies.

Recombinant allergens: allergenic molecules that have been produced in vitro by recombinant deoxyribonucleic acid (DNA) techniques.

Variable region: the antigen-binding portion of an immunoglobulin molecule.

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