

Advances in IgE Testing for Diagnosis of Allergic Disease



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Date of Original Release: September 1, 2020. Credit may be obtained for these courses until August 31, 2021.

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Overall Purpose/Goal: To provide excellent reviews on key aspects of allergic disease to those who research, treat, or manage allergic disease.

Target Audience: Physicians and researchers within the field of allergic disease.

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List of Design Committee Members: Robert G. Hamilton, PhD, Wolfgang Hemmer, PhD, Anna Nopp, PhD, and Jörg Kleine-Tebbe, MD (authors); Michael Schatz, MD, MS (editor)

Learning objectives:

1. To apply molecular diagnostic methods for allergy diagnosis.
2. To discuss advantages and limitations of current IgE testing modalities.
3. To incorporate patient history and prior probability reasoning to interpret tests of IgE sensitization.

Recognition of Commercial Support: This CME has not received external commercial support.

Disclosure of Relevant Financial Relationships with Commercial Interests: W. Hemmer has received nonfinancial support from ThermoFisher Scientific; and personal fees from MacroArray Diagnostics, outside the submitted work. J. Kleine-Tebbe reports personal fees from AllergenOnline (Nebraska, USA), Allergy Therapeutics, Allergopharma, ALK-Abelló, AstraZeneca, Bencard, Dr. Pfleger, HAL Allergy, InfectoPharm, LETI, Merck US, Sanofi Genentech, Springer International Publishers, ThermoFisher Scientific, and Thieme Publishers, Germany; has received grants and personal fees from GSK, Lofarma, Novartis, and Stallergenes-Greer; has received nonfinancial support from American Academy Allergy Asthma and Immunology, European Academy of Allergy and Clinical Immunology, and World Health Organization/International Union of Immunological Societies Allergen nomenclature subcommittee; and has received personal fees and nonfinancial support from the German Society of Allergy and Clinical Immunology; all outside the submitted work. The rest of the authors declare that they have no relevant conflicts of interest. M. Schatz declares no relevant conflicts of interest.

Since its discovery in 1967, IgE antibody detection in skin and blood has identified a state of allergic sensitization and served as a necessary but not sufficient risk factor that requires objective

symptoms to make the definitive diagnosis of human allergic disease. More recently, quantitative IgE antibody levels in serum against allergenic extracts, molecules, and epitopes have pushed

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No funding was received for this work.

Conflicts of interest: W. Hemmer has received nonfinancial support from ThermoFisher Scientific; and personal fees from MacroArray Diagnostics, outside the submitted work. J. Kleine-Tebbe reports personal fees from AllergenOnline (Nebraska, USA), Allergy Therapeutics, Allergopharma, ALK-Abelló, AstraZeneca, Bencard, Dr. Pfleger, HAL Allergy, InfectoPharm, LETI, Merck US, Sanofi Genentech, Springer International Publishers, ThermoFisher Scientific, and Thieme Publishers, Germany; has received grants and personal fees from GSK, Lofarma, Novartis, and Stallergenes-Greer; has received nonfinancial support from American Academy of Allergy Asthma and Immunology, European

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Received for publication July 13, 2020; revised July 21, 2020; accepted for publication July 22, 2020.

Available online July 24, 2020.

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2213-2198

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<https://doi.org/10.1016/j.jaip.2020.07.021>

Abbreviations used

ALEX- Allergy Explorer

BAT- Basophil activation test

CV- Coefficient of variation

CCD- Cross-reactive carbohydrate determinant

ISAC- Immunosorbent Allergen Chip

WHO- World Health Organization

its application into more accurately identifying the specificity of the allergic response for targeting immunotherapy, predicting allergic symptom severity after allergen exposure, and attempting to distinguish tolerance from food allergy. This review examines new *in vivo* and *in vitro* developments in the design, performance, interference, and application of the methods used to identify allergic sensitization. The increasing accepted applications of molecular allergen and allergen epitope-based IgE antibody measurements, especially as applied to food allergy diagnosis and management, are highlighted as state-of-the-art advances. Despite these major advances in allergic sensitization documentation, their ultimate value requires integration by the clinician with the patient's history and pretest probability of disease. © 2020 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2020;8:2495-504)

Key words: Allergen; IgE antibody; Molecule-based allergy diagnosis; Performance; Quality assurance; Type 1 hypersensitivity; Human; Multiplex; Microarray; Serodiagnosis; Singleplex

Allergic sensitization as defined by the presence of an allergen-specific IgE antibody in the skin or blood is a key risk factor used in the diagnosis of human allergic disease. It alone, however, is insufficient to definitively make the diagnosis without a direct linkage with an objective clinical history of allergic symptoms that are temporally associated with an allergen exposure.¹ Since 1967 when IgE was identified as the fifth human immunoglobulin isotype and the gatekeeper of the allergic response,² there have been significant improvements in the clinician's and laboratory's ability to detect and quantify the IgE antibody's presence using *in vivo* and *in vitro* methods, respectively. This review will examine the current state of the art in methods for the detection of a human IgE antibody. It will place in evidence the strengths and weaknesses of each method's ability to quantify the 4 attributes of an IgE antibody response³: concentration, epitope specificity, strength of binding, or affinity and its specific activity (specific to total IgE ratio). This overview will not examine cell-based assays such as the basophil activation test (BAT) as discussed elsewhere^{4,5} because the BAT and associated basophil assays remain important research-based assays that are performed in limited immunology laboratories, but rarely used as routine diagnostic tests. This is due among other reasons to the need for fresh blood and a concern regarding the approximately 10% of inherent nonresponders. Newly developed technologies will be examined that allow precise dissection of the humoral immune response to allergens with less specimen and thus make enhanced accuracy and speed of IgE quantification a present day reality.

DISSECTING THE IgE ANTIBODY RESPONSE

Of the 4 fundamental measures of the humoral immune response,³ IgE antibody specificity was the first attribute to be extensively documented in atopic individuals using *in vivo* provocation methods that used allergen extracts in early qualitative skin test⁶ and radioallergosorbent test analyses.⁷ It has principally been with immunoassays and rarely with *in vivo* provocation tests that the specificity of the IgE antibody analysis has been enhanced with the use of molecular allergenic components⁸ and more recently allergenic epitopes.⁹ Enhanced analytical specificity has allowed more effective monitoring of allergenic cross-reactivity, risk assessment for severe allergic reactions, and improved prediction of unresponsiveness after immunotherapy particularly in food allergy. With technological improvements, the concentration of the IgE antibody in skin and serum became the next attribute to be successfully measured using *in vivo* skin semiquantitative skin test titration¹⁰ and more commonly with quantitative singleplex autoanalyzer-based serological IgE antibody immunoassays traceable to the World Health Organization (WHO) IgE reference preparation.¹¹ Moreover, it has only been with the availability of these quantitative immunoassays¹² of total and specific IgE that the measurement of the third parameter of the humoral immune response (the specific IgE/total IgE ratio) has allowed more accurate prediction of the success of omalizumab treatment vis-à-vis allergic reactions.¹² The fourth immune response parameter (avidity/affinity)³ has been the only elusive humoral immune response parameter that is not easily measured in routine clinical laboratory settings with existing assay methods.

IN VIVO PROVOCATION ASSAYS

From its earliest use in 1921,⁶ local itching, swelling, and erythema after the puncture or intradermal injection of an allergen extract into the skin of a sensitized individual has remained possibly the most powerful and rapid diagnostic procedure for the assessment of allergic sensitization. Visualization of a wheal and erythema larger than a saline control reaction signals to the patient and clinician allergic sensitization and the risk of an immediate-type hypersensitivity reaction possibly more convincing than a clinical immunology laboratory-based measurement of the specific IgE antibody. Visual evidence of a wheal and erythema after the administration of an extract to the patient's skin supports the clinical history-based diagnosis of human allergic disease.

Despite its long history of use and widespread acceptance, prick/puncture and intradermal skin testing have remained a medical art with its associated variability resulting from a general lack of standardized allergen extracts, the use of a multiplicity of skin testing devices and application techniques, interference from selective drugs, and interoperator scoring and interpretation variability of the final wheal and erythema response.¹³⁻¹⁵ Evaluation of various skin test devices confirms the inherent variation in skin test responses that can occur using the same extract with different puncture devices.^{16,17} A novel short-wave infrared camera technology has been applied to skin test reading that improves reproducibility and allows more uniform reading of skin test responses, especially in pigmented skin.¹⁸ Otherwise, there have been few recent developments to improve skin test performance. Finally, skin testing techniques are not completely risk free, and they may sometimes not be well tolerated,

especially by children. For this reason, serum-based assays for the detection of allergen-specific IgE antibodies have become important in the diagnostic algorithm as an equivalently effective means to demonstrate allergic sensitization.

Regarding *in vivo* challenge tests, conjunctival and nasal provocation tests are particularly useful when the clinical history is vague and allergen-related symptoms need to be confirmed by a challenge procedure.¹⁹ Bronchial provocation challenges with allergens remain primarily research procedures that are used to identify a relationship between an inhaled substance and a change in the patient's bronchial pathophysiology. The food challenge remains the most widely used challenge test particularly for children. Details of these procedures are discussed elsewhere.²⁰

ALLERGEN EXTRACT AND MOLECULE-BASED SEROLOGICAL IMMUNOASSAYS

In 1996, the Clinical Laboratory Standards Institute convened an international body of scientists from academia, industry, and the US Government to establish a consensus guidance document (ILA20).²⁰ This reference has served to foster harmonization and enhance the analytical performance of IgE antibody detection methods that are performed in diagnostic immunology laboratories throughout the world. Since then, the ILA20 has been updated in 2009 and 2016 to reflect enhancements in the state of the art using new solid phases (eg, fluorescence embedded particles, magnetic beads, chips, nanoporous cassettes), native and recombinant molecular allergens⁸ to supplement aqueous allergen extracts,¹⁴ improved computer-driven automation, and novel detection modalities.²⁰ The document examines technical aspects and performance requirements for federal licensure of laboratory-based diagnostic allergy methods including but not limited to specimen sources, preparation and validation of allergen and IgE detection reagents, assay calibration, quality control and external proficiency strategies, and optimal performance expectations required by various regulatory agencies. The ILA20 document's principal goal has been to promote harmonization of methods, so someday IgE antibody measurements performed using different assay systems could be directly comparable. Quantitative total serum IgE assays have achieved this mark of excellence with intermethod comparability as evidenced by persistent interlaboratory coefficients of variation (CVs) <10% for the quantitative autoanalyzers and intermethod CVs <15%, as reported by a US-based external proficiency program involving over 150 clinical laboratories.²¹ In contrast, allergen-specific IgE assay results as measured by different autoanalyzer-based assays continue to exhibit larger than desirable interassay variability, even to the present day.^{21,22} The reasons for this are believed to stem largely from the use of different heterogeneous allergen extracts and calibration algorithms. Whether the use of an identical source of allergen extracts or purified allergen molecules in the different autoanalyzer-based IgE antibody assays will reduce this observed variability remains to be determined.

Even if the use of identical molecular allergens improves IgE antibody intermethod variance, the allergen extract will in general remain the ideal source of allergenic material for assays used in the initial evaluation of patients for allergic sensitization. There may be certain cases where it may be more cost-effective to begin serological testing directly using allergosorbents containing

molecular components when the history-determined allergen specificities are identified as those (eg, birch, cat, ragweed, and peanut) that are the major allergens for their specificity and they are known to be highly predictive of a specific sensitization. Considering the complexity of allergen extracts, which commonly contain multiple allergenic molecules with varying clinical potency, and the heterogeneity of individual IgE sensitization profiles, the predictive power of such quantitative IgE measurements may be expected to be higher and better comparable between centers when single, well-defined components are used instead of extracts. In most cases, however, it will be the heterogeneous allergen extract that provides the most cost-effective and comprehensive source of major and minor allergen components in any of the over 500 clinically important allergen specificities.

Level of quantification/calibration

There are a number of significant contributions that the ILA20 consensus document has provided to the *in vitro* diagnostic allergy field.²⁰ Possibly most important has been its classification of IgE antibody assay methods based on their degree of quantitation (Table 1). Different assays have been assigned to different levels of performance based on their design and their ability to accurately quantify the level of IgE antibody to a given allergen specificity.²⁰ For instance, newer dot blot and lateral flow point of care assays that provide only a *qualitative* positive/negative result are less analytically sensitive and more variable than computer-driven autoanalyzer-based assays. This contrasts with the original radioallergosorbent test and newer chip-based multiplex assays that provide interpolated results from an internal calibration curve into *semiquantitative* estimates of the IgE antibody as classes or grades. These tend to provide a relative ranking of IgE levels, often with improved analytical sensitivity over the qualitative assays. The highest performing and reproducible assays, which are mostly autoanalyzers, produce *quantitative* measures of the IgE antibody in kU_A/L units. Their overall performance (interlot and laboratory precision) is exceptional. The autoanalyzers can be standalone floor units such as the ImmunoCAP, Immulite, and Noveos or desktop units such as the Abioscope and Luminex. They are capable of analyzing sera for the IgE antibody to extracts, molecules, and most recently epitopes with excellent reproducibility and analytical sensitivity to 0.1 kU_A/L (0.24 ng/mL) provided by computer-driven interpolation and automation. Importantly, the ability to dilute out in parallel (parallelism) must be maintained between the calibrator/standard and unknown specimens to ensure that the assay system is quantitative.

One of the major contributions of the reproducible quantitative measurements of the specific IgE antibody has been the ability to define the relationship between a given IgE antibody level in serum and the probability that a patient will experience an allergic reaction after an allergen exposure (dose vs reaction probability prediction curves).²³ Heterogeneity due to subject selection, variable cohorts, and study designs have hampered reproducible, uniform threshold levels for reliable prediction and ultimate elimination of controlled or open food challenges.

Specimen requirements

Serum and plasma remain the principal working specimens for almost all total and allergen-specific IgE antibody immunoassays. Newer point of care assays such as the Abioscope and

TABLE I. Classification scheme for allergen-specific IgE assays

Classification	Result presentation	Standardization method	References calibrators and controls
Qualitative	• Reactive or nonreactive • Positive or negative • An “indeterminate” zone may be included	• Single or dual calibrators to normalize assay	• One reference sample • Negative and positive control
Semiquantitative	• Arbitrary units or classes	• Single or dual calibrators to normalize assay	• Negative and bilevel positive controls (occasionally trilevel controls) • References unique for the test system made from pooled patient sera
Quantitative	• Units related to a generally recognized IRP (eg, WHO IgE standard 75/702)	• Multipoint standard curve used for interpolation	• Trilevel positive controls • References calibrated towards a qualified reference preparation

IRP, International reference preparation; WHO, World Health Organization.
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cassette-based lateral flow assays use whole blood collected from a finger stick. Alternative specimens that have also been analyzed include tear fluid, bronchial and nasal lavage fluid, cerebrospinal fluid, sputum, and cell supernatants.²⁰ There are issues with so-called matrix effects when using these different specimens, and these effects can be controlled by analyzing comparable specimens from healthy subjects to rule out differential nonspecific binding. The specimen volume requirement has also become an important differentiating variable as newer systems such as the Noveos and Abioscope successfully use 4 μ L of human blood (serum or capillary whole blood) with no apparent loss in assay performance. This is contrasted with the required 40 μ L of specimen needed in addition to a dead volume for established quantitative singleplex autoanalyzers (ImmunoCAP, Immulite). When evaluating pediatric patients, a 4 μ L or capillary blood sample becomes attractive as it permits a maximum number of specific IgE antibody measurements that can be performed from a single collection.

Calibration and unit considerations

Most clinical immunoassays that measure discrete analytes (drugs, hormones, chemotherapeutic agents) use a purified standard identical to the analyte being measured to generate a reference curve for interpolation of unknown response data into defined concentrations. In the field of diagnostic allergy, this was tried in 1994 with the Abbott Matrix,²⁴ and it failed to be adopted as the universal strategy for IgE antibody assay calibration. The reason stems from the difficulty in obtaining sufficient volumes of IgE antibody containing sera and validating them for each of the hundreds of IgE antibody specificities of clinical importance. A more practical calibration system was, however, adopted in which specific IgE antibody response data are interpolated from a “heterologous” universal total IgE reference curve that is traceable to the WHO IgE Reference Preparation. As long as the total serum IgE and allergen-specific IgE dilution curves remain parallel with each other,²⁰ this “heterologous interpolation” scheme²⁵ remains a valid and efficient calibration system for the allergen-specific IgE antibody immunoassays of different specificities.

Although each of the available Food and Drug Administration—cleared quantitative IgE antibody autoanalyzers used in clinical laboratories in North America generates results across a working range of 0.1 to 100 kU_A/L, laboratories use various lower limits of detection to report their results to

clinicians. This has led to confusion when a clinician who is used to seeing undetectable IgE antibody levels reported as <0.1 kU_A/L receives a report from a different laboratory that uses the <0.35 kU_A/L limit of detection. In 2015, a plea was made to all clinical laboratories to report down to the method’s regulatory agency agreed on lower limit of detection that in North America is 0.1 kU_A/L.²⁶ On the basis of proficiency surveys, some laboratory staff still use 0.35 kU_A/L as their limit of detection because they believe that levels <0.35 kU_A/L are clinically unimportant. However, the decision of what level of IgE antibody is clinically relevant must be made by the clinician, taking into account the patient’s symptom history, and not the laboratory staff. For instance, Linden et al²⁷ reported 0.2 kU_A/L as the optimal cutpoint based on receiver operator characteristics curves using a Yojuden’s J index for teens exhibiting allergic symptoms after dog allergen exposure.

Immunoglobulin isotype

Reaginic allergen-specific IgE antibody is the primary analyte measured in diagnostic allergy laboratories to assess allergic sensitization. In research environments, allergen-specific IgG/IgG1/IgG4 antibodies are measured to monitor increases in humoral immune responses that directly block IgE binding to allergen and inhibit IgE-dependent activation that is mediated through both high-affinity IgE receptors on mast cells and basophils and low-affinity IgE receptors on B cells.²⁸ More recently, allergen-specific IgA antibodies have increasingly been of interest for their potential blocking effects as measured in research studies of food allergy. The Stanford group for instance has characterized IgE+ B-cell clones in blood, stomach, duodenum, and esophagus of peanut-allergic patients. They found that IgE+ cells in allergic patients were enriched in stomach and duodenum, and they provided evidence for local isotype switching, including transitions between IgA and IgE isotypes.²⁹

Serum factor interference in IgE assay analysis

Until recently, assay performance (degree of quantification, analytical sensitivity, and reproducibility) and analytical accuracy (closeness of measurement to the true result) have been the important factors that laboratory staff consider when selecting an IgE antibody assay method for patient testing. Other factors such as required sample volume, cost, and availability of reagents have generally been secondary issues. However, in recent years, there

TABLE II. Allergen families and their associated biological functions

Family	Function	Examples
Profilins	• An actin-binding protein involved in the dynamic turnover and restructuring of the actin cytoskeleton • A highly conserved and ubiquitous protein in pollen and plant (foods) inducing broad cross-reactivity	Bet v 2 (birch), Phl p 12 (Timothy grass), Amb a 8 (Ambrosia), Hev b 8 (natural rubber latex), Mal d 4 (apple)
Serum albumins	• Plasma protein carrier involved in transport of hormones, enzymes, hemin, and fatty acids • Also maintains oncotic pressure	Fel d 2 (cat), Can f 3 (dog)
Pathogenesis-related 10 protein family (PR-10), Bet v 1 homologs	• Plant defense proteins • Bet v 1 is aquercetin-3-O-sophoroside-binding molecule (17 kDa) • Inflammation response proteins	Bet v 1 (birch), Cor a 1 (hazelnut), Mal d 1 (apple), Gly m 4 (soy)
Polcalcins	• A calcium-binding protein involved in calcium regulation in pollen inducing broad cross-reactivity	Phl p 7 (Timothy grass), Bet v 4 (birch), Amb a 10 (short ragweed)
Nonspecific lipid transfer proteins (nsLTP) (PR-14)	• Inflammation response protein in plants • Responsible for shuttling phospholipids and other fatty acids between cell membranes	Ara h 9 (peanut), Cor a 8 (hazelnut), Pru p 3 (peach)
Lipocalins	• An extracellular protein involved in transport of small hydrophobic molecules such as steroids, bilins, retinoids, and lipids	Fel d 4, Fel d 7 (cat), Can f 1, Can f 2, Can f 4, Can f 6 (dog)
Parvalbumins	• A calcium-binding protein • Localized in fast-contracting muscles that are involved in calcium signaling	Gad c 1 (cod), Cyp c 1 (carp), Gal d 8 (chicken)
Tropomyosins	• An integral component of actin filament that plays a role in regulating muscle contraction • Also regulates actin filament stability in nonmuscle cells	Der p 10 (dust mite), Pen m 1 (shrimp)
Seed storage protein	• A heterodimeric protein involved in nutrient storage, eg, 2S albumin, 7/8S globulin (vicilins), 11S globulin (legumins)	Ara h 2, Ara h 1, Ara h 3 (peanut), Cor a 14, Cor a 11, Cor a 9 (hazelnut)

PR, Pathogenesis related.

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has been increased interest in possible assay interference by factors in blood that alter IgE antibody assay accuracy. Human serum factors that can produce false-positive results include heterophilic antibodies such as IgM and IgE rheumatoid factors that cross-link detection antibodies with allergen-specific non-IgE antibodies.^{30,31} Depletion of these anti-human autoantibodies has been shown to be important in demonstrating the role of IgE antibodies in drug-related allergic reactions.³²

In addition, IgE anti-cross-reactive carbohydrate determinant (IgE anti-CCD) that binds to carbohydrate-based solid phases such as those used in the agarose-based assays and the ImmunoCAP have been shown to produce false-positive IgE results.^{33,34} A solution has been proposed to this internationally discussed concern of IgE anti-CCD producing false-positive results in the assays that use carbohydrate allergosorbent matrices. It involves adding synthetic CCD blocker to the assay buffer to reduce the number of CCD-related false-positive IgE antibody results without compromising the sensitivity of relevant IgE antibody interactions with the allergosorbent.^{35,36}

Serum factors that can produce false-negative results include exogenous biotin supplements taken orally as a supplement that interferes with biotinylated-allergen binding avidin-coated particles.^{37,38} Finally, omalizumab (therapeutic anti-IgE) differentially influences the accuracy of multiple IgE antibody assays.³⁹ Difficulties in accurately measuring total serum IgE after omalizumab administration remains problematic even with so-called free IgE assay methods.⁴⁰ Finally, high levels of allergen-specific IgG antibody blocking induced after chronic natural or immunotherapy-induced allergen exposure can block IgE antibody binding to allergosorbents, especially on microchips such as the Immunosorbent Allergen Chip (ISAC) where the allergen density is limited.^{41,42}

For the most part, these interferences have been overlooked by laboratory's staff who perform IgE testing and by clinicians who interpret IgE antibody results in caring for their patients. The principal reason is that the extent of interference and the actual presence of these interfering factors are difficult to predict and interference requires elaborate reanalysis schemes.

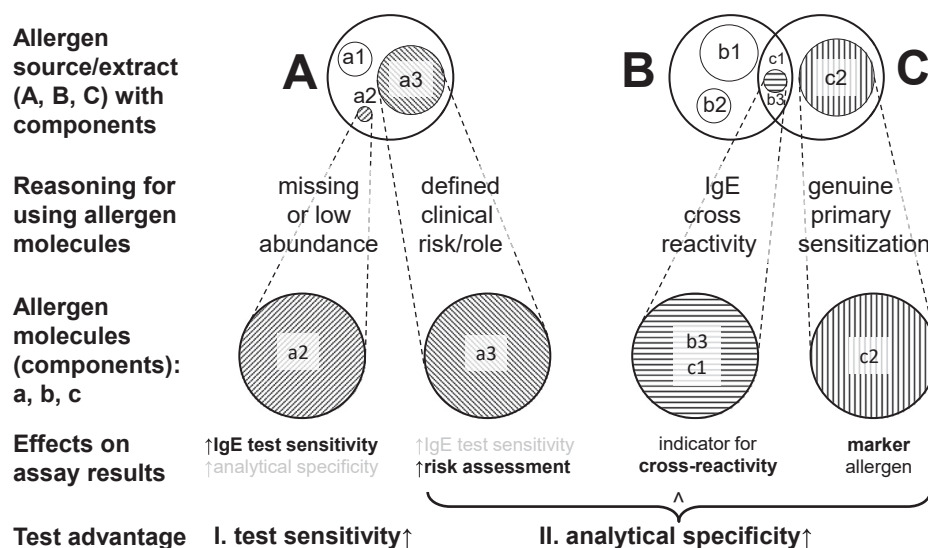


FIGURE 1. Utility of allergen molecules as reagents for specific IgE antibody testing.

TABLE III. Examples of the utility of allergen molecules as reagents

Examples (allergen source/carrier)	Increased test sensitivity Optimized LLoQ	Defined clinical risk/role	Cross-reactive allergens	Species-/family-specific marker allergens
Cat		Fel d 2 (inducer of cat-pork-syndrome)	Fel d 2	Fel d 1
Hazelnut	Cor a 1 (Bet v 1-homolog)	Cor a 14 (2S albumin) Cor a 9 (11S globulin) Cor a 8 (LTP, Mediterranean)		
Kiwi	Act d 8 (Bet v 1-homolog)		Act d 8 (Bet v 1-homolog)	
Peach	Pru p 1 (Bet v 1-homolog)	Pru p 3 (LTP, marker, Mediterranean)	Pru p 1 (Bet v 1-homolog), Pru p 4 (Profilin)	
Peanut	Ara h 10, Ara h 11 (oleosins)	Ara h 1 (7S globulin) Ara h 2 (2S albumin) Ara h 3 (11S globulin) Ara h 6/7 (2S albumin) Ara h 9 (LTP, Mediterranean)	Ara h 8 (Bet v 1-homolog), Ara h 5*	
Soy	Gly m 4 (Bet v 1-homolog)	Gly m 4 , Gly m 5 , Gly m 6 , Gly m 8 (2S albumin)		
Wheat	Tri a 19 (omega-5-gliadin)			
Meat	alpha-GAL	alpha-GAL		
Bee venom	Api m 3 , Api m 4 , Api m 10	Api m 1 , Api m 3 , Api m 4 , Api m 10		Api m 1 , Api m 3 , Api m 4 , Api m 10
Wasp venom	Ves v 5	Ves v 1 , Ves v 5		Ves v 1 , Ves v 5
Birch (hazel, alder, birch pollen) and beech trees (beech, oak pollen)		Bet v 1	Bet v 2 *, Bet v 4 †	Bet v 1
Oleaceae (ash, olive pollen)		Ole e 1	Ole e 2 *, Ole e 3 †	Ole e 1
Poaceae (pollen from moderate climate grasses)		Phl p 1 , Phl p 5	Phl p 12 *, Phl p 7 †	Phl p 1
Mugwort pollen		Art v 1	Art v 4 *, Art v 5 †	Art v 1
Ragweed pollen		Amb a 1	Amb a 8 *, Amb a 10 †	Amb a 1

LLoQ, Lower limit of quantification; LTP, lipid transfer protein.

Bold letters: available for IgE testing; normal letters: not (yet) available for IgE testing.

*Profilin (panallergen in pollen and plant foods).

†Polcalcin (panallergen in pollen, see Table II for definitions).

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NOVEL TRENDS IN IgE ANTIBODY SENSITIZATION ASSESSMENT

Point of care testing

Strategies for bringing testing to the doctor's office for rapid patient testing were seriously considered in 2009 and then abandoned due to concern over its lower analytical sensitivity and fixed aeroallergen panel than available for singleplex autoanalyzers. A prototype point of care lateral flow device called the ImmunoCAP Rapid displayed a limit of detection depending on the allergen specificity from 0.7 to 2.6 kU_A/L.⁴³ It was designed to detect the IgE antibody to 10 aeroallergen specificities. In this assay, serum proteins from capillary whole blood migrate up 2 membranes each with 5 aeroallergens immobilized. If present, allergen-specific antibodies bound to the immobilized allergen and subsequently colloidal gold-labeled anti-IgE detected bound IgE antibody. An external reader was used to detect and report positive responses in semiquantitative units.

However, there is renewed interest with the emergence of new assay methods, and it has new support from pharmacy chains and drug manufacturers. These newer tests are miniaturized, handheld devices or miniature tabletop autoanalyzers with a complex chemistry that has been simplified by computer-based automation. It involves the manual addition of a finger prick whole blood drop to a plastic cassette. The tabletop autoanalyzer⁴⁴ uses centrifugal force to move capillary blood through a nanofluidic chambers in a cassette containing immobilized molecular allergen and labeled anti-IgE. These assays are designed to produce a rapid analytical result with a qualitative (positive/negative) or quantitative estimate of the IgE antibody to 1 or multiple allergen specificities. They have improved the analytical specificity of the generated measurements due to the use of major allergen components instead of extracts. The goal of this point of care strategy is more widespread sensitization testing for aeroallergen-related allergic disease, which can then be followed up by consultation with board-certified clinicians for management.

Chip-based assays

Molecular allergology has ushered in a number of chip-based microarray assays for allergen-specific IgE. Some use only molecular allergens,⁴⁵ whereas others use a combination of extract and molecular components immobilized on the chip.⁴⁶ The ISAC is a microarray with 112 allergenic components that has been evaluated in patients with nut allergy. The positive detection rates of skin prick tests (56%) and ISAC (65%) were lower than those of singleplex ImmunoCAP (71%). In anaphylaxis to wheat, the ImmunoCAP test results identified all 9 patients, whereas the ISAC was positive in only 6 of these 9. In contrast, the Allergy Explorer (ALEX) is a macroarray in which 157 allergen extracts and 125 molecular components are immobilized. Serum is mixed with CCD Inhibitor to minimize CCD interference with extracts and glycosylated purified components and then added to the chip, followed by labeled antihuman IgE. Although the 2 chip-based assays differ in methodology and the use (or not) of allergen extracts, the IgE profiles detected were reportedly similar between the ISAC and ALEX. The optimal application for these assays resides in epidemiology studies where they can define the atopic status of different populations in geographically diverse regions. Their analytical sensitivity is generally less than singleplex autoanalyzers

and their unitage is semiquantitative. Because of the low density of allergen on the chip dots, nanogram levels of IgE antibody binding can be interfered with in the ISAC by the presence of microgram quantities of allergen-specific IgG "blocking" antibodies.⁴⁷ Possibly most relevant to clinical testing, chip-based microarrays have fixed allergen panels and thus are not as versatile as singleplex systems at covering all the important allergen specificities. Even the ALEX that uses some extracts cannot compete with the singleplex autoanalyzers that have comprehensive allergen extracts immobilized on larger solid phase matrices. Because of the higher cost of the chip array, the testing of fixed allergen specificities (some that may not be relevant to the particular patient), the semiquantitative nature of the chip-based assay results, and the more restricted allergen specificity panels, these molecular component and extract-based microarrays and macroarrays will remain excellent research and tertiary center expert tools but probably not become first-line diagnostic tests for patients. The possible exceptions are those patients who have very complex sensitization profiles and where chip-based panel testing may more effectively distinguish primary from cross-reactive sensitizations.⁴⁸

Molecular allergen-based IgE antibody assays

Use of molecular allergens in the singleplex autoanalyzers has been possibly the most important single technological advance for diagnostic allergy serology in the recent years. The European Academy of Allergy and Clinical Immunology Molecular Allergy User's Guide⁸ provides the first practical and comprehensive reference on the clinical applications of allergenic molecules. The guide presents the clinically important allergenic molecules within the context of their protein families based on their common structures and function (Table II). The use of molecular allergens in IgE antibody assays has been extensively reviewed,^{49,50} and they offer several important advantages, as illustrated in Figure 1 and Table III. First, allergen extracts that have low or missing allergens as a result of their extraction procedure can be supplemented or replaced with a recombinant form of the low abundance allergen. This lowers the limit of quantitation and makes the assay more analytically sensitive because the IgE antibody with specificity for the originally missing allergenic molecule can now be detected (Figure 1, A). Examples where this supplementation process has been important include hazelnut, kiwi, peach, and peanut extracts where Cor a 1, Act d 8, Pru p 1, and Ara h 10/11, respectively (Table III), are low or missing after an aqueous extraction. Second, the analytical specificity of the antibody assay can be enhanced by measuring IgE antibody responses to proteins (i) that signal a certain risk for serious allergic reactions or (ii) that serve as exemplified indicators of broad cross-reactivity (due to panallergen-specific IgE), or (iii) that represent a marker allergen, mostly major allergens of one particular allergen source (Table III). The best illustration for (i) involves peanut in which strong IgE positivity to the stable seed storage proteins Ara h 1, Ara h 2, and Ara h 3 identifies a high probability for a serious allergic reaction. In contrast, an exclusive IgE antibody response to Ara h 8, the labile and barely present Bet v 1 homolog (Table III), signals a low risk if any when exposed to peanut.⁴⁸ Focusing on (ii), the use of molecular allergens such as PR10 proteins Bet v 1, birch profilin Bet v 2, or birch polcalcin Bet v 4 separates IgE antibody binding to highly cross-reactive pan-allergens (Figure 1, B) that otherwise would not be identifiable if

the respective complex protein extract for birch pollen was used for diagnostics, particularly in pollen-polysensitized individuals. In the latter case, well-selected molecular allergens such as Amb a 1, Art v 1, Bet v 1, Ole e 1, and Phl p 1 can serve as reliable species-/family-specific marker allergens for their specificity (Figure 1, C). IgE antibodies to these proteins are able to confirm sensitization to *Ambrosia* species, *Artemisia vulgaris*, *Fagales* trees, olive/ash pollen, and moderate climate grasses (*Pooideae*), respectively (Table III).

Allergen epitope-based assays

An epitope is defined as the minimum molecular structure of the antigenic site, be it linear or conformational, that will react with a monoclonal antibody. Since the late 1990s, Sampson et al have been systematically mapping IgE antibody linear and conformational epitopes on allergenic molecules in cow's milk (eg, casein, alpha-lactalbumin, beta-lactoglobulin), egg (ovomucoid), the 2S albumin in cashew (Ana o 3), and the 11S globulins in hazelnut, English walnut, and peanut (Ara h 1, Ara h 2, Ara h 3) with the goal of identifying differences that might signal a persistent versus transient food allergy that results in tolerance. In one of their many early studies,⁵¹ they probed dot-blots containing linear (denatured) and conformational (native) milk proteins with sera from milk-allergic children who became either clinically tolerant or had persistent cow's milk allergy. They also probed synthesized decapeptides from alpha(S1) and beta casein on membranes that they believed from previous work to be predictive of a persistent cow's milk allergy. Both analyses led them to conclude that children with persistent milk allergy had higher levels of IgE antibodies to linear epitopes from casein than children who achieved tolerance. Importantly, IgE antibodies to amino acids 69-78 from alpha(S1) casein could be a predictor for persistent cow's milk allergy. Fast forward to today's work in which their blots and membrane studies have evolved into a state-of-the-art Luminex bead-based epitope multiplex immunoassay in a CLIA-88 licensed clinical laboratory that allows IgE antibody mapping to tolerance or allergy predictive epitopes involving multiple foods.⁵² This unique service needs to be more thoroughly evaluated with sera from food-allergic and tolerant children who have undergone food challenges to verify that epitope-specific IgE measurements can address the problem of low diagnostic specificity of food extract-based singleplex autoanalyzer measurements that have resulted in the overdiagnosis of food allergy for decades.

Allergen phage library-based microarrays to identify immunodominant epitopes

There is a growing literature on the application of phage display coupled with protein microarrays for the identification of IgE-reactive proteins, components, peptides, epitopes, and mimotopes in a range of allergenic molecules including but not limited to Bet v 1, Ara h 1, Ara h 2, Ara h 6, tropomyosin, Hev b 6.02, and Bla g 2. One illustrative group constructed a T7 phage display library of mustard seed and olive pollen and biopanned it with sera from mustard and olive allergic patients.⁵³ They then constructed a phage microarray printed with over 1500 monoclonal phages from their previous repetitive biopanning studies. These phage microarrays were then screened with other sera from mustard seed and olive pollen-allergic patients. Ten and 9 IgE-reactive unique linear amino acid sequences from olive pollen and mustard seed, respectively, were identified. Five

immunoreactive amino acid sequences displayed on phages were expressed as His6-GS tag fusion proteins and validated as allergenic epitopes by further IgE antibody binding studies. This work illustrates a T7 bacteriophage-dependent technique that is being increasingly used to identify clinically important immunodominant allergenic epitopes.

In a novel modification of this technique, Monaco et al⁵⁴ reported on a programmable T7 phage display-based assay to analyze allergen-specific IgE antibody epitopes to 1857 allergenic proteins with overlapping 56 amino acid peptides. They downloaded the complete library of allergenic proteins from the Allergome database (called AllerScan Library) and input it into their T7 Phage Immunoprecipitation Sequencing pepsin library design pipeline to obtain 19,334 "56 amino acid peptide tiles" with 28 amino acid overlaps. This was encoded by a library of synthetic 200-mer oligonucleotides, and finally amplified and cloned into the T7 phage display system for automated IgE antibody profiling. These details are presented to illustrate how allergen epitope identification has become highly sophisticated. Importantly, this technique allows analysis of longer, higher quality peptides for allergen epitope searching than has been possible with the synthetic peptide microarrays of the past. Using the AllerScan approach, a novel IgE binding epitope that appears to discriminate between wheat allergy and sensitization/tolerance was identified.

Allergy diagnostic practice parameters

Even today, the most comprehensive treatise on diagnostic allergy testing is the practice parameter prepared by Bernstein et al in 2008.⁵⁵ It covered every aspect of *in vivo* and *in vitro* methods for diagnosing human allergic disease, albeit with the assay and reagent technology available at that time. Since 2008, there have been major developments in reagents and methods used in diagnostic allergy testing. More specifically, the procurement, characterization, and production of allergens has been revolutionized by the use of molecular biology-based recombinant and phage methods and more precise analytical instrumentation to document allergenic structure and purity.¹⁴ Likewise, developments involving new solid phase chip and particles materials, enhanced protein coupling chemistries, novel enzyme and fluorescence conjugates, enhanced signal detection instrumentation, miniaturized computer-driven autoanalyzer and nanotechnology-based devices, enhanced hybridoma methods to produce naturally occurring human IgE monoclonal antibody standards,⁵⁶ and a new (third) WHO IgE reference preparation have all changed the landscape of serological IgE antibody detection methods.

In an attempt to update the diagnostic practice parameter from 2008 to include modern technology, the requirement that new practice parameters are required to have a Cochrane-type systematic literature review and meta-analysis has all but ensured that we will never have a single global practice parameter update as comprehensive as the Bernstein et al document. Such a requirement limits any future diagnostic practice parameter to an in-depth study of diagnostic methodology associated with essentially one analyte specificity such as peanut or hazelnut. To attempt the process of preparing a prototype Cochrane meta-analysis-based practice parameter, 2 initial meta-analyses have been prepared. One involves peanut and its components,⁴⁸ and the second is focused on hazelnut and its components.⁵⁷ These 2 food allergen specificities were selected because of the clinical

importance of their components (eg, Ara h 1, Ara h 2, Ara h 3 and 8; Cor a 1, Cor a 8, Cor a 9, Cor a 14) and provides clarity to extract-based IgE antipeanut and hazelnut extract measurements. They also illustrate the power of allergenic component-specific IgE in clarifying allergic responses to these foods in children. It is, however, clear that the effort and cost of such limited practice parameters pales to the utility of the Bernstein et al style evidence-based practice parameter.

CONCLUDING THOUGHTS

In recent years, assays for the IgE antibody have become more diagnostically specific with the use of allergenic components and epitopes and more analytically sensitive with newer chemistries, more well-designed automated instrumentation, and the availability of more highly quality controlled reagents. It is unclear the role the results of qualitative and semiquantitative point of care and unlicensed assays will have in providing support to the clinicians who have access to highly sophisticated governmental-validated autoanalyzers that provide quantitative IgE antibody measurements that allow some predictions related to disease risk and severity. However, the clinical relevance of these allergic sensitization assay results that define the concentration, specificity, and specific activity of allergen-specific IgE in the patient's skin or blood remains independent of the use of allergen extracts, molecules, or epitopes. This is because their ultimate value in the overall diagnostic algorithm can ultimately only be determined by the physician's interpretation with facts from the patient's history and the pretest probability of disease and not solely by the test itself.⁵⁸

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