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In vitro assays for the diagnosis of IgE-mediated disorders

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Advances in technology have provided new laboratory tools for the quantitation of allergen-specific IgE antibodies in serum and on the surface of basophils. This review examines the evolution from qualitative IgE antibody assays of the late 1960s to the present-day, third-generation, automated and quantitative allergen-specific IgE assays. The latest technology trend is toward microarrays in which crude or purified native and recombinant allergens can be spotted in microdot arrays on silica chips to permit extensive panels of specific IgE measurements to be performed with small quantities of serum. Although these technologies hold promise, their diagnostic performance requires further assessment once their technical details have been optimized. Potential abuses of this newer IgE antibody technology include the use of allergosorbent specificities (eg, especially food and drugs) that lack validation, application of IgE antibody measurements in the diagnosis of non-IgE-dependent disorders (eg, aspirin sensitivity), and modification of IgE antibody assays to measure food-specific IgG antibody for which there is no clinical indication. Basophil mediator release assays have evolved to include flow cytometric methods that can quantitatively detect the presence of cell surface-bound allergen-specific IgE antibodies. Assays for histamine and leukotriene C₄ released after *in vitro* basophil activation are now more accurate and standardized. Current analytic methods for IgE antibodies provide more quantitative and reproducible measurements of IgE than ever before, although still with less sensitivity than traditional skin testing. The current challenge is to translate the quantitative IgE antibody results into a more accurate diagnosis of allergic disease. (*J Allergy Clin Immunol* 2004;114:213-25.)

Key words: *IgE antibody, diagnosis, serologic assays, basophil, histamine, sulfidoleukotriene, CD63, CD203c, allergy*

The diagnostic algorithm for human allergic disorders (immediate or type 1 hypersensitivity) begins with

Abbreviations used

ASR: Analyte-specific reagent
CAST: Cellular Antigen Stimulation Test
CLIA-88: Federal Clinical Laboratory Improvement Act of 1988
FAST: Flow assay stimulation test
FDA: US Food and Drug Administration
LTC₄: Cysteinyl leukotriene C₄

a thorough clinical history and physical examination. Temporal associations between allergic symptoms and allergen exposures lead to a high degree of suspicion that the patient has an allergic disorder. Once this inference has occurred, confirmatory testing for IgE antibody is often performed to strengthen the probability that the working diagnosis is correct. This may involve *in vivo* methods (skin or other provocation testing) or laboratory-based *in vitro* analyses. This review focuses on laboratory-based *in vitro* serum- and cell-based technologies that are now available for the assessment of IgE-mediated disorders (Table I). The traditional wheal-and-flare skin test remains the gold standard for IgE antibody detection largely because of its unexcelled sensitivity. *In vitro* alternatives offer numerous advantages that can include precise quantitation, absolute safety, a lack of drug interference, and, for serologic tests, long-term storage of specimens. Advancing technologies are increasing the attractiveness and cost-effectiveness of multiple and simultaneous *in vitro* assessments of IgE antibodies but also the potential for misuse.

SERUM-BASED TECHNOLOGIES FOR DETECTION OF ALLERGEN-SPECIFIC IgE ANTIBODY

Our ability to quantify allergen-specific IgE antibody in serum and on the surface of cells began in the late 1960s with the identification of human "reagin" or "skin-sensitizing antibody" as IgE.¹ The availability of purified

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TABLE I. *In vitro* diagnostic tests for the diagnosis of IgE-mediated disorders

Specimen source	Analyte	Advantages	Limitations
Serum	IgE antibody assays for individual allergen specificities	1. Second- and third-generation immunoassays provide quantitative IgE antibody results. 2. Automation: increased precision and shorter turnaround times. 3. Miniaturization chip technology reduces serum requirement. 4. Ability to repeat IgE analyses with stored serum for longitudinal assessment. 5. Adaptable for use with purified native and recombinant allergens.	1. Delayed results in comparison to skin testing (1+ -day turn around). 2. Insufficient analytic sensitivity for some allergies (venoms, drugs, and latex). 3. Potential antigenic competition and isotype (IgG) inhibition.
Serum	Multiallergen specific IgE antibody screening assays	Single test for qualitative detection of IgE to principal aeroallergen and food specificities	1. Designed best to confirm nonatopic status. 2. Allergen specificities on multiallergen screen not defined and different among various manufacturers. 3. Different reagents required for children and adults and for individuals in different countries.
Whole blood or leukocyte preparation	Basophil mediator release assay. 1. Histamine release assay (eg, BASO Test). 2. Cysteinyl leukotriene assay (CAST)	Simple, rapid assay	1. Not useful in nonreleasers (10%-15% of subjects). 2. Less sensitive than skin testing leading to false-negative results. 3. Antigen extract usually uncharacterized. 4. Optimal antigen concentration is basophil (patient) dependent. 5. Requires processing of blood within 24 h.
Leukocyte preparation	Cytometric Basophil activation assay (flow CAST). 1. CD63 flow-based assay. 2. CD203c flow-based assay.	1. Simple, rapid assay. 2. CD203c flow assay design is simpler than CD63 flow assay because of basophil restriction.	1. Less sensitive than skin testing, leading to false-negative results. 2. Requires processing blood within 24 h. 3. Potential for false-positive results from IL-3 priming. 4. Criteria for positivity vary with antigen specificity.

human IgE myeloma protein permitted the preparation of the IgE Fc fragment and the production of polyclonal antibodies specific for the ϵ heavy chain. With human IgE-specific polyclonal animal antisera available, the first total serum IgE radioimmunosorbent test and allergen-specific IgE RAST were developed (Table II).²⁻²³ The radioimmunosorbent test used insolubilized purified anti-human IgE to capture serum IgE and radiolabeled anti-IgE in a second incubation to detect bound IgE. In contrast, the first RAST used allergen coupled to a cyanogen bromide-activated Sephadex allergosorbent to bind allergen-specific antibodies of all isotypes from serum. After a buffer wash to remove unbound serum proteins, bound human IgE antibodies were detected with a radiolabeled anti-human IgE Fc.²

This initial Sephadex-based RAST assay was cumbersome for routine use in the clinical laboratory. It was therefore modified into a more laboratory-friendly assay (the Phadebas RAST) that used a cyanogens bromide-activated paper disc allergosorbent (Fig 1).^{3,24} A birch-specific IgE calibration curve allowed response data (counts per minute bound) to be interpolated into estimates of the relative level of IgE antibody in serum. These first-generation IgE antibody assays were generally semi-quantitative, and several scoring methods were developed to organize results into 4 to 6 classes that still persist today. They have undergone major improvements in all aspects of their technology that have resulted in present-day, quantitative, second-generation IgE assays and the emerging third-generation, automated IgE antibody assays.

TABLE II. Past and current serologic assays for allergen-specific IgE in North America

Assay method	Allergosorbent	Chemistry	Assay generation	Status*†	References
Phadebas RAST	CNBr-activated paper discs	NC-SP-IM	1	FDA cleared	2, 3, 10-13
Cellulose RAST	CNBr- activated microcrystalline, cellulose particles	NC-SP-IM	1	Research	4
Agarose RAST	CNBr-activated Sepharose CL-4B particles	NC-SP-IM	1	Research	5
FAST fluorescent allergosorbent test	Allergens	NC-SP-IM	1	Withdrawn	23
CLA (MAST)	Hitachi (formally MAST Immunosegments) threaded pipette	NC-SP-IM	1	FDA cleared	20
CAP System	Pharmacia: sponge SP	NC-SP-IM	2	FDA cleared	6, 9, 13-15, 17
Magic Lite	Corning-ALK	NC-SP-IM	1	Withdrawn	22
Matrix	Abbott Laboratories	NC-SP-IM	2	Withdrawn	21
AlaSTAT	Diagnostic Products Corp (LA)	NC-LP-IM	2	FDA cleared	7, 8
Immulin 2000	Diagnostic Products Corp (LA)	NC-LP-IM	3	FDA cleared	19
UniCAP System	Pharmacia: sponge SP	NC-SP-IM	3	FDA cleared	6, 16, 18

CNBr, Cyanogen bromide; NC, noncompetitive; SP, solid phase; IM, immunometric (labeled antibody); LA, labeled antigen; LP, liquid phase.

*The designation "FDA cleared" in this table means that the assay method has been technically shown to be comparable with a preexisting predicate device (typically the Phadebas RAST). It does not indicate that the diagnostic performance (sensitivity and specificity) of the assay has been verified by the FDA or that it is deemed acceptable.

†In addition to clearance of the assay method, each allergen specificity (allergosorbent) that is used as a distinct reagent in an assay is separately reviewed by the FDA for performance data and clearance. If an allergosorbent has not achieved FDA clearance but has been quality controlled with limited human sera from individuals known to have an allergy to that allergen specificity, it can be given an ASR (analyte-specific reagent) status and used in a clinical laboratory that is CLIA certified for highly complex testing, providing an appropriate disclaimer is provided on the laboratory report.

Improvements in several components of the assays have evolved.

Allergen-containing reagent and binding chemistry

The solid-phase (allergosorbent) or liquid-phase allergen reagent is the principal component of the assay that confers specificity on the IgE antibody assay. It is the most complex and highly variable reagent in IgE antibody assays, in part because of the heterogeneity of most allergen extracts and the different chemistries used to insolubilize or label the allergenic proteins. In an attempt to improve on the antibody-binding capacity of the paper disc, a variety of carbohydrate-based allergosorbents (other than Sephadex and paper), such as microcrystalline cellulose and agarose, were historically used in research (Table II).^{4,5} The most significant advance for clinical assays, however, was the development of an encapsulated hydrophilic carrier polymer to which allergen was covalently coupled.⁶ This polymer was configured into the shape of a small cup and called a CAP. Its allergenic protein-binding capacity was both superior to the paper disc and more user friendly than agarose or cellulose particles. Its use in the Pharmacia CAP system improved the allergosorbent's overall antibody-binding capacity, which led to more rapid assay kinetics and an enhanced assay sensitivity. Diagnostic Products Corporation chose to label allergenic protein with biotin.⁷ This permitted rapid solution phase binding of allergens to antibodies in serum and subsequent insolubilization on an avidin bead. Concordance of positive food-specific IgE antibody results between these 2 assay systems was 80%.⁸

The complex nature of allergen extracts provides the greatest challenge to optimization of IgE assays.

Immunochemists have spent decades identifying so-called major allergens, which have been described as antigens to which a majority of allergic individuals produce IgE antibody.²⁵ Once identified, the next step has been to clone the gene, sequence it, and then produce recombinant allergens in unlimited quantities. Although progress is being made, it will be years before the principal allergens of even the most important allergen groups are identified and recombinant allergens are generated. Recombinant allergens promise continuous and reproducible quantities of allergenic proteins in purified form for use in *in vitro* assays or for immunotherapy. One recent illustration of the importance these recombinant allergens can have in IgE antibody assays involves the major natural rubber latex allergen *Hevea brasiliensis* 5 (Hev b 5).^{26,27} This acidic protein is labile and potentially lost when natural rubber latex extracts are insolubilized as allergosorbents. Purified recombinant Hev b 5 has been used to enrich natural latex extracts before allergosorbent preparation, resulting in detection of higher levels of IgE antibody and, more importantly, increased diagnostic sensitivity of Pharmacia CAP assay for latex-specific IgE by 10%, with no loss in diagnostic specificity.⁹ Whether other IgE immunoassays can be engineered to achieve higher sensitivity relative to skin testing by means of supplementation of low-level but important allergens with recombinant sources remains to be studied.

A second important development has involved proteomics and the adaptation of chip technology originally applied to nucleic acid hybridization assays.²⁸ The chip platform has permitted the development of prototype miniaturized IgE antibody assays that use microarrays of allergenic proteins attached to an activated silica chip.²⁹⁻³¹ With this technology, small quantities (eg, 10 nL) of

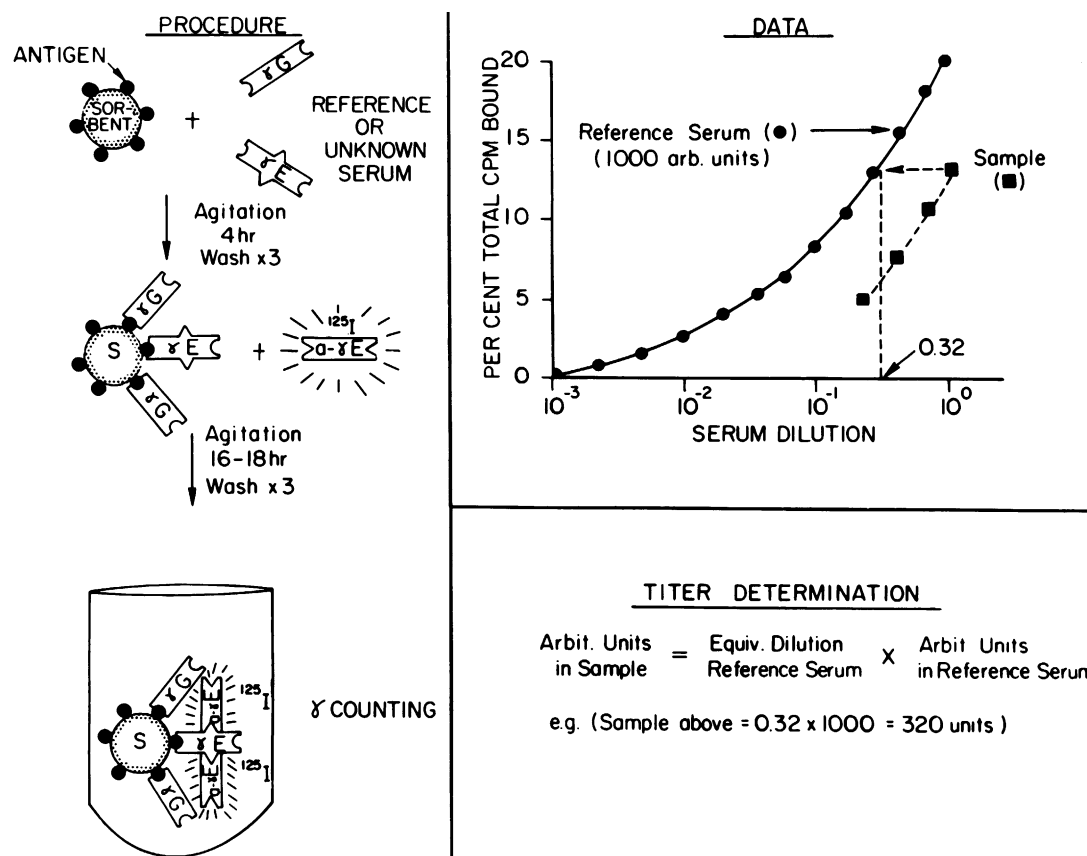


FIG 1. Schematic diagram of the original RAST for IgE antibody developed in 1967. In the first incubation allergen-specific antibody is extracted from serum with an allergosorbent. After buffer washes to remove unbound serum proteins, bound IgE antibody is detected with an iodine 125-labeled rabbit anti-human IgE Fc detection antibody. The counts per minute (CPM) bound to the allergosorbent are proportional to the amount of specific IgE in the original serum. Even in this earliest assay, quantitative IgE antibody results were emphasized. The test serum IgE antibody results were obtained at multiple serum dilutions, interpolated from a multipoint dose-response curve, corrected for dilution, and reported in units relative to the quantity of IgE antibody in the original serum. Reproduced with permission from Adkinson.²⁴

purified or crude mixtures of allergenic proteins are uniformly spotted in triplicate on activated silica chips by using a robotic microsprayer at concentrations of 1 mg/mL. The ordered array with defined spacing (eg, 1500 μm) theoretically permits thousands of allergens to be attached to a microchip surface. One consequence is that minimal volumes of serum are needed for detection of IgE to many allergen specificities. Biotin-labeled anti-human IgE and Cy3-conjugated streptavidin are finally used to detect bound human IgE antibody on the chip, and the bound anti-IgE is quantified by scanning the spin-dried chip by using a specially designed fluorescent microscope.²⁹

A number of proof-of-concept studies have investigated the use of crude or purified native or recombinant allergens in microarray systems with varying success. Kim et al²⁹ attached to silica chips unpurified *Der-matophagoides pteronyssinus*, egg white, milk, soybean, and wheat allergen extracts and human serum albumin as a negative control. Purified human IgE at varying concentrations was bound in nonoverlapping spots on

the same chip to establish an IgE (kIU/L) dose versus luminescence response calibration curve. When 2-fold dilutions of IgE anti-*D pteronyssinus* were simultaneously analyzed in the chip-based IgE assay and Uni-CAP System, the chip IgE Assay displayed an analytic sensitivity of 1 kIU/L, which was 3-fold less sensitive than the 0.35 kIUa/L achieved in the CAP System.²⁹ Interpolated *D pteronyssinus*-specific IgE levels, as measured in 10 sera from dust mite-sensitive patients by using both the chip and CAP assays, were positively correlated, but the concordance of semiquantitative grade levels (grade 0 = negative, grades 1-7 = increasingly positive) was higher ($r = 0.97$; $P < .05$). The authors showed that crude allergens spotted on silica chips can bind IgE antibody. However, the report provides little conclusive data about the accuracy and precision of chip IgE assay results with crude allergen extracts other than *D pteronyssinus*. Furthermore, specificity was not systematically studied. More general applications of chip-based IgE assays will require further study to look for aberrant results and to improve sensitivity if possible.

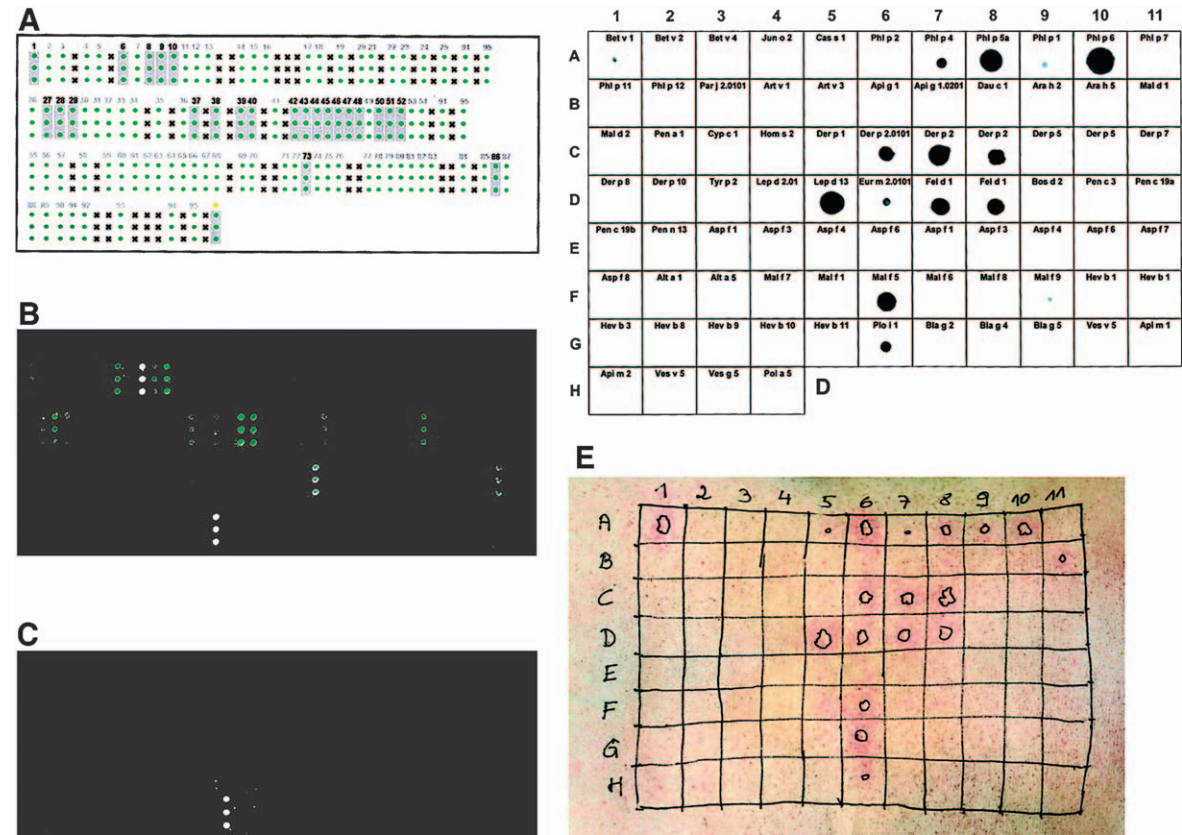


FIG 2. Allergen microarray. **A**, Scheme of environmental allergens spotted in triplicate in vertical columns. **B** and **C**, Scanned images from a microarray chip-based IgE assay obtained with serum from an allergic (Fig 2, B) and nonallergic (Fig 2, C) individual. **D**, Dot-blot IgE reactivity to the same allergens bound to nitrocellulose. **E**, Skin reactivity to allergens and histamine (H6; wheal circled for visualization). Reproduced with permission from Hiller et al.³⁰

Because the microspots on the chip have such a small protein-binding capacity, the use of purified native allergens, recombinant allergens, or both may improve the chip-based IgE assay's performance. An international collaborative study has provided proof-of-concept for the use of microarray technology in the detection of allergen-specific IgE antibody by using purified native and recombinant allergens.³⁰ Recombinant ($n = 78$) and native ($n = 16$) allergen molecules representing major allergen groups, including mites, animal epidermals, fungi, insect venoms, and tree, grass, and weed pollens were microarrayed onto glass chips and exposed to 40 μ L of human serum from 20 allergic patients with defined allergen sensitivities. Bound IgE antibody was detected with a fluorescent-labeled monoclonal anti-human IgE. Although the clinical and assay performance data presented in this article are limited, the authors concluded that IgE immunodots on nitrocellulose paper compared well with the observed chip-based IgE antibody reactivity profile (Fig 2). There was, however, no observed correlation between the magnitude of the puncture skin test reactions to purified allergens and fluorescence intensity measured in the microarray chip IgE assay.

In a more recent study the analytic performance of a proteomics chip IgE assay was compared with that of the

Pharmacia CAP system and a research microtiter plate-based ELISA with a panel of purified recombinant grass pollen (Phl p 1, 2, 5, and 6) and tree pollen (Bet v 1 and 2) allergens.³¹ The coefficients of variation for results of replicates of the same allergens ranged from 25% to 47%. The authors reported comparable analytic sensitivities for the proteomics chip, the Pharmacia CAP assay, and an ELISA IgE assay using end point dilution analysis³² as their method of comparison. Interestingly, for Phl p 6-specific IgE detection, the chip IgE assay was analytically more sensitive than the CAP method.

Miniaturization with chip technology and purified allergens has distinct advantages and disadvantages. Advantages include a smaller serum requirement for the chip assay that could allow broad-spectrum allergy evaluations with finger-stick blood samples, a boon for pediatric patients. The chip-based immunoassay has the potential for complete automation, with the benefit of reduced assay variation, more precise quantitation by using multiple replicates, and a more rapid turnaround time. The activated silica chip can easily accommodate large numbers of allergen specificities (theoretically several thousands) in triplicate, but such comprehensive testing is likely to remain expensive because of the cost of native or recombinant allergens. On the negative side,

some allergens used as mixtures or highly charged allergens may not spot well on silica chips. Also, antigenic competition among cross-reactive allergens will confound quantitation and could reduce diagnostic sensitivity. Competition of IgG and IgE antibodies for limited quantities of spotted allergens will also be a concern, especially for foods and in patients with current or previous immunotherapy. Lastly, any fully automated, large-capacity IgE-detection system will likely lead to the inclusion of unvalidated specificities, as we have already seen happen, especially in food and drug allergen testing.

Calibration and quantification

By the early 1980s, a number of technical concerns about the RAST were noted that made it less clinically attractive compared with skin testing.^{10,11} These included the delay of days to weeks in obtaining results, a limited menu of allergen specificities relative to clinical needs, and increased costs of testing compared with skin tests. Another concern was the number of arbitrary RAST data-reporting schemes that made comparison of results difficult. The Phadebas RAST used a 5-point birch-specific IgE reference curve from which allergen-specific IgE levels in test sera were interpolated in arbitrary Phadebas RAST units per milliliter.¹² In an attempt to extract more analytic sensitivity from the RAST, a problematic modified scoring system was created.³³ It used a buffer negative control and a single 35 kIU/L total serum IgE-positive time control to construct a 2-point dose-response curve. This led to an appreciable rate of false-positive results.^{13,34} Another concern was competitive inhibition of IgE antibody binding to limited insolubilized allergen by IgG antibody in serum from individuals with high-dose natural exposure or allergen immunotherapy. Finally, the analytic sensitivity of the original RAST appeared to vary as a function of the allergenic proteins coupled to the allergosorbent. All of these concerns led us to the conclusion that “we are not yet in 1981 at the point for the routine use of RAST testing for clinical diagnosis.”¹¹

Emergence of the second-generation IgE antibody assays, the paradigm of which was the Pharmacia CAP System introduced in 1990, brought with it a new heterologous calibration scheme and quantitative reporting.^{6,29,35} A total serum IgE dose-response curve was used to interpolate unknown allergen-specific IgE response data into kilo international units of allergen-specific IgE antibody per liter, where 1 IU was equivalent to approximately 2.4 ng of IgE. Dilution curves of individual IgE antibody specificities to their respective allergosorbents could be shown to be parallel to the heterologous total serum IgE reference curve, thereby validating the method.²⁵

The quantitative results from these second-generation IgE antibody assays has allowed investigators to study whether the quantity of serum IgE antibody has any predictive utility in defining clinical sensitivity. The

approach has been most rewarding. In the area of food allergy, several groups have now shown that the quantity of specific IgE antibody in serum to peanut, egg white, cow's milk, and fish can accurately define a patient's current clinical sensitivity, as determined diagnostically with double-blind, placebo-controlled food challenges.^{14,15,36} Sampson^{14,15} has published for 5 foods the probability distribution for a positive food challenge as a function of food-specific IgE antibody in serum using the Pharmacia CAP assay. By using probability curves, it is possible to define IgE thresholds for provocative testing below which there is a greater than 95% probability that the challenge result will be negative. Alternatively, upper threshold limits define IgE levels above which a challenge test is more than 95% likely, thereby avoiding the need for this cumbersome, expensive, and sometimes uncomfortable clinical procedure (Fig 3).

Soderstrom et al¹⁶ have extended the use of probability-based risk assessment to respiratory allergy using quantitative allergen-specific IgE antibody data previously reported from 4 European laboratories. The authors used a logistic regression model to compare the relationship between the doctor's final diagnosis of allergic respiratory disease (positive or negative) on the basis of the clinical history, physical examination, skin testing and serologic data and the quantitative level of serum IgE antibody alone. Probability curves were calculated in this study to show the relationship between IgE antibody in blood and the dichotomous clinical diagnosis of the absence or presence of allergic respiratory disease. Fig 4 displays the probability of obtaining a positive allergy diagnosis at a given serum IgE antibody level with the Pharmacia UniCAP System for different allergens at different clinics. Differences in the shape of the IgE antibody level versus probability of clinical disease curves were seen both between allergens within a clinic and between clinics for the same allergen specificity. This indicates that use of specific IgE antibody levels to support the clinical diagnosis of allergic disease is different for the same allergist depending on the particular inhalant allergen and between allergists for the same allergen specificity. Importantly, however, the authors make the case that quantitation of serum IgE antibody improves the confidence of the clinical diagnosis of inhalant allergies better than simply knowing whether IgE antibody is present or absent.

Niederberger et al³⁷ came to a different conclusion with regard to the clinical utility of quantitative serum IgE antibody measurements in the diagnosis of respiratory allergy. They used purified recombinant timothy grass and birch pollen allergens to compare the relative ability of puncture skin testing, nasal provocation, and IgE antibody serology by means of the CAP System to reflect immediate-type respiratory sensitivity. Although the skin test and nasal provocation results were significantly correlated, the intensity of these biologic reactions did not correlate with the level of allergen-specific IgE antibody in serum. The authors concluded that factors in addition to IgE influence the extent of allergic tissue reactions. In their study skin testing provided a better

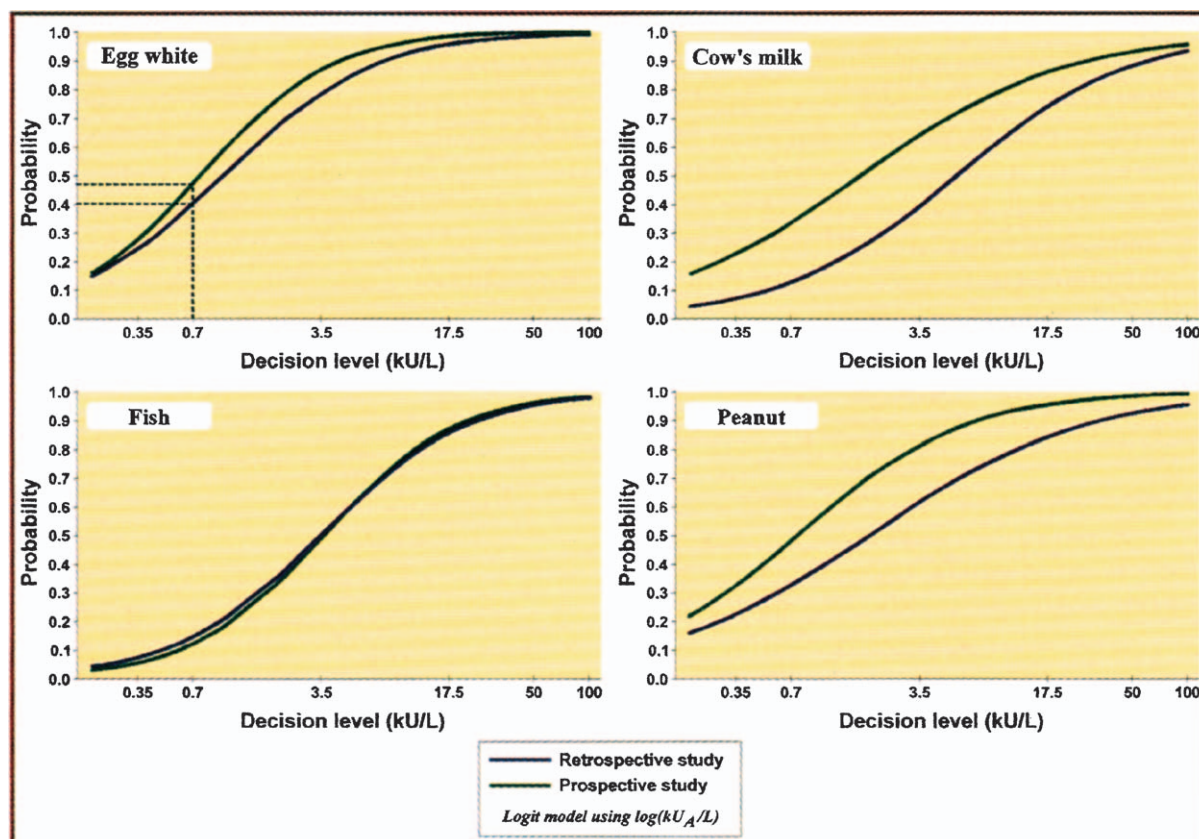


FIG 3. Probability curves from a retrospective and prospective study of patients reacting to 4 foods (egg white, cow's milk, fish, and peanut) given a measured quantitative level of allergen-specific IgE antibody. The food allergen-specific IgE 95% decision level (kUa/L) above which a double-blind, placebo-controlled food challenge is not considered necessary is as follows: egg, 7 kUa/L; milk, 15 kUa/L; peanut, 14 kUa/L; and fish, 10 kUa/L. Not shown in the figure are the 95% decision levels for soy (65 kUa/L) and wheat (80 kUa/L). Reproduced with permission from Sampson.¹⁵

reflection of magnitude of an immediate-type respiratory sensitivity to inhaled allergens than did serologic IgE measurements. This result is of course not contradictory to the work of Soderstrom et al.¹⁶

Finally, regarding the diagnosis of Hymenoptera venom sensitivity, serology has recently become a complementary diagnostic test to the skin test for those patients who experience a systemic reaction in the face of a negative intradermal skin test result. The American Academy of Asthma, Allergy and Immunology practice parameters were modified in 2003 to recommend IgE anti-venom serology in cases in which a positive clinical history is not confirmed by means of intradermal venom skin testing.^{17,38} The reason why IgE serology may be more sensitive than intradermal skin testing in some cases is not currently known.

Utility of IgE antibody screening assays

In cases in which the clinical indication for atopic disease is weak, a single qualitative multiallergen screening assay for IgE antibody to multiple allergen specificities can support the absence of allergic disease.³⁹ In these

assays a panel of up to 15 allergen specificities representing those primarily involved in aeroallergen-induced allergic disease are coupled to a single allergosorbent. The allergen specificities attached to the allergosorbent may vary for children and adults; for individuals in Europe, Asia, and North America; and between manufacturers. The results of these screening assays can be highly predictive of individual IgE antibody results obtained by using a panel of separate skin tests or *in vitro* IgE antibody tests. Screening assays, such as the Phadiatop (Pharmacia), provide a positive or negative result. If negative, it may be the single best test for confirming the absence of significant atopic disease in individuals who are suspected of having an intrinsic or non-IgE-mediated disease process. The negative predictive value of this single test is higher than total serum IgE or any single specific IgE antibody measurement for identifying non-atopic individuals. Such a test can minimize the need for multiple *in vivo* or *in vitro* allergen-specific IgE measurements in patients with a low clinical probability of atopic disease. Another application has been in the evaluation of children to identify the potential for allergic disease. In one study the combined aeroallergen and food allergen

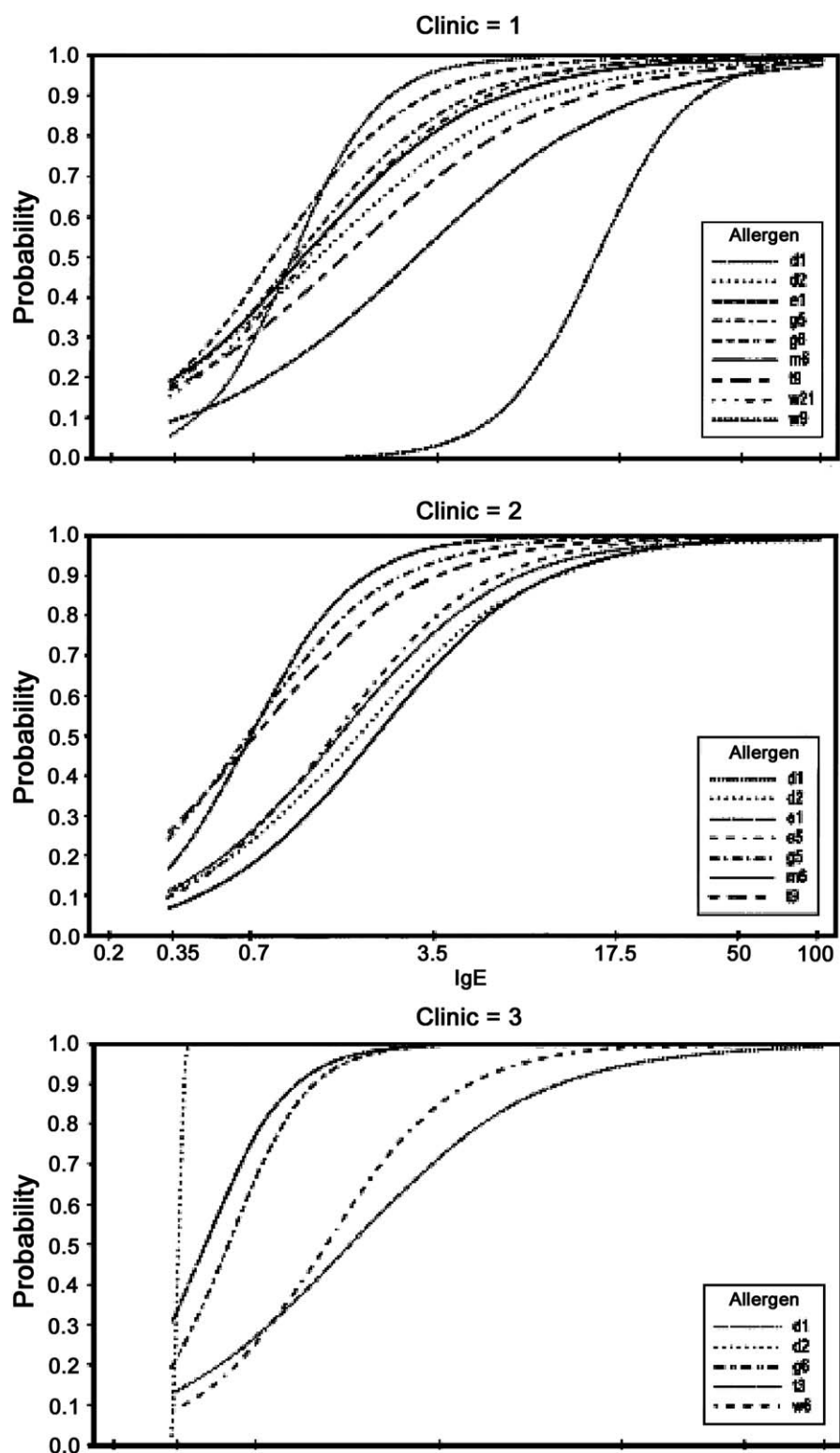


FIG 4. Selected curves demonstrating the probability of receiving a positive allergy diagnosis at a given IgE antibody level for different allergens at 3 different clinics. Each curve's shape demonstrates the allergist's disposition for a positive clinical allergy diagnosis in relation to the level of allergen-specific IgE antibody in serum. The different slopes indicate different identification patterns of symptoms. A flat slope reflects difficulty in linking high IgE antibody levels as a trigger for allergic symptoms. A steep slope indicates that allergic symptoms are easily linked to low levels of allergen-specific IgE antibody. Extracted and reproduced with permission from Soderstrom et al.¹⁶

screening assays for IgE antibody (Phadiatop and Fx5-CAP System) not only were able to predict allergic disease in 24% of 4-year-old children, but the presence of IgE antibody also related to severity of such diseases as asthma.⁴⁰ There was a poor correlation between sensitivity to aeroallergens and food allergens in young children. Caution, however, should be exercised when using screening tests in unselected populations because they can generate false-positive results as a result of IgE antibody responses being more frequent than symptomatic disease.

Third-generation assays

Currently, there are 5 assay methods used clinically to detect allergen-specific IgE in human serum in North America (Table I). On the basis of data from the College of American Pathologist's Diagnostic Allergy Proficiency Survey,⁴¹ 3 of these, the chemiluminescent assay from Hitachi Chemical Diagnostics (formally MAST), the Hycor Hy-Tech EIA (formally the Ventrex RAST), and the Thabest IgE, are infrequently used first-generation technologies that report qualitative (positive-negative) or semiquantitative (class-grade) results. The Pharmacia CAP system and the Diagnostic Products Corporation Alastat are second-generation assays that have achieved a high degree of quantitation, semiautomation, and good analytic performance. In recent years, both of these assays have transitioned to stand-alone, push-button, automated third-generation systems known as the Pharmacia UniCAP and Diagnostic Products Corporation Immulite systems, respectively.^{18,19} Automation permits tighter precision around the positive-negative threshold and a more rapid and robust chemistry. However, because the analysis system has essentially become a black box into which the serum and component reagents are input and out of which results are generated by a computer, there is an increased need for quality control with established positive control sera with defined IgE antibody-binding ranges. These quality control sera should have IgE antibody levels across the measured dose-response curve range, and they should be randomly sprinkled through the assay run to investigate the quality of the whole assay analysis. Daily monitoring of results from these positive control sera in the form of a Levey-Jennings plot verifies that the instrument is performing all of its serum and reagent addition, incubation, washing, and data analysis steps accurately.

Evaluation of IgE antibody testing laboratories

The quality of allergen-specific IgE antibody measurements reported from clinical diagnostic allergy laboratories is not uniformly equivalent.⁴² The clinician requesting IgE antibody tests has some responsibility for determining whether testing is performed in a clinical laboratory that is federally licensed for highly complex immunology clinical testing under the Clinical Laboratory Improvement Act of 1988 (CLIA-88). Information should also be obtained on the assay method used and the source of its reagents. Occasionally, large clinical laboratories manu-

facture their own allergosorbents, with sometimes less than ideal quality control programs. Lastly, the conscientious clinician will also request details of the laboratory's internal quality assurance program. Levey Jennings plots of quality control data should be provided by any reputable laboratory, as well as records on the laboratory's external proficiency testing program over the past year. Currently, in North America the College of American Pathologists conducts external proficiency surveys involving IgE analysis of 5 or 6 sera every 17 weeks.⁴¹ Finally, the clinician should require that the assay method and IgE antibody units are defined on the final report and ask about the use of analyte-specific reagents (ASRs). Most diagnostic allergy laboratories will print a disclaimer at the bottom of their report indicating that a test on the report has been performed with an ASR. The ASR designation means that a particular allergosorbent's analytic performance has been defined by its manufacturer but it has not been cleared by the US Food and Drug Administration (FDA). This occurs because the manufacturer can technically not obtain sufficient numbers of IgE-positive sera from characterized patients with history and skin test data confirmation to validate the allergosorbent on the basis of FDA requirements. For rare allergen specificities, sera from a sufficient number of allergic patients are simply not available for validation studies. Before reputable manufacturers release these allergosorbents, however, each is generally validated with at least several characterized human sera that are known to contain relevant allergen-specific IgE antibody.

CELL-BASED TECHNOLOGIES FOR DETECTION OF ALLERGEN-SPECIFIC IgE ANTIBODY

Activation of the secretory response in human basophils in the presence of IgE cross-linking doses of allergen has been extensively used by research laboratories as an alternative measure of allergen-specific IgE antibody.^{43,44} However, to date, these assays have not been widely used in the diagnosis of allergic patients for a number of reasons. The assays involve whole blood, which needs to be processed within 24 hours. Cell-based assays are time consuming and expensive, and they require special technical skill. Not all sensitized individuals release mediators from their basophils on allergen challenge.^{45,46} And possibly most important, the reagents and assay methods have not been standardized. Recently, a number of commercially available assays for mediators have begun addressing this issue of assay standardization. The assays with the most potential as alternative diagnostic confirmatory tests for IgE-dependent allergic disorders are discussed.

Allergen-induced mediator release assays

Allergen cross-linking of IgE on the surface of basophils induces the release of a number of mediators,

including histamine and cysteinyl leukotriene C₄ (LTC₄). In both the basophil histamine and LTC₄ release assays, heparinized whole blood is incubated with increasing concentrations of anti-IgE or allergen. *In vitro* stimulation is usually performed in the presence of IL-3 to augment sensitivity. After incubation, the cells are centrifuged, and the supernatants are collected and either frozen or analyzed for histamine or LTC₄ content by means of immunoassay. The net level of mediator release is computed by subtracting the level of spontaneous release from the allergen-induced mediator release.

Histamine release

The criterion for defining a positive basophil histamine release varies among the different studies from 1% to 20% of total histamine release, depending on the allergen specificity and concentration used.^{45,47,48} Over the past 10 years, at least 7 published studies have examined the performance of the basophil histamine release assay in light of clinical history and skin test results. The diagnostic sensitivity of the basophil histamine release in comparison to skin provocation testing, inhalation provocation testing, or both ranged from 50% with β -lactam drugs⁴⁸ and yellow jacket venom⁴⁹ as the challenging allergen to 94% with inhalant allergens, such as house dust mite, cat, and dog extracts.⁵⁰ The diagnostic specificity observed with basophil histamine release relative to skin testing in these controlled studies ranged from 43% with Hymenoptera venom⁴⁹ to 90% with inhalant allergens.⁵¹ The data from these studies support the conclusion that the basophil histamine release assay, although generally useful for discriminating between IgE-sensitized and nonsensitized individuals, is still not as diagnostically efficacious as skin testing.

LTC₄ release

An assay method for measuring LTC₄ released from allergen-activated basophils has been commercialized as the Cellular Antigen Stimulation Test (CAST)-ELISA by Buhlmann Laboratories in Europe and distributed in North America by American Laboratory Products Corporation.⁵² Selected allergen extracts verified to be nontoxic to basophils are available, and the LTC₄ assay sold by Buhlmann for use with either whole blood preparations or washed leukocytes is robust and analytically sensitive. Over the past 6 years, 4 clinical studies have compared the predictive value of the CAST with that of skin testing by using dust mite, food, Hymenoptera venoms, and drugs as challenging allergens. The observed diagnostic sensitivity of the CAST compared with the combination of a clinical history and skin test ranged from 18% with aspirin⁴⁹ to 85% for selected food allergens.⁵³ The reported diagnostic specificity of the CAST in the same studies ranged from 67% to 100%. From these data, we conclude that the CAST assay is not sufficiently sensitive for effective use in the diagnosis of IgE-mediated sensitivities to β -lactam or nonsteroidal anti-inflammatory drugs. Its utility in the diagnosis of sensitization to other

allergen specificities appears more promising, but further clinical studies are warranted.

Current utility as diagnostic tests

Despite their undisputed value as research procedures, the basophil histamine and LTC₄ release assays are rarely used today in the routine diagnosis of human allergic disease. It is unlikely that this will change in the foreseeable future for a number of reasons. In addition to the need for rapid blood processing and the concern about non-releasers, these *in vitro* test results add little to the diagnostic predictive value offered by skin and provocation testing. Moreover, the performance of mediator release assays reportedly varies with the quality of allergen extracts available and the techniques (concentrations, incubation times, criteria for positivity, quality control reagents, and methods) used among laboratories.

Flow cytometric basophil activation assays

In the late 1990s, basophils were shown to upregulate the expression of a number of surface proteins (eg, CD45, CD63, CD69, and CD203c) when activated by allergen.⁵³⁻⁵⁵

CD63 is a member of the transmembrane-4 super family that is expressed on basophils, mast cells, macrophages, and platelets.⁵⁶ It is attached to intracytoplasmic granules in resting basophils from both healthy and allergic individuals. IgE-sensitized basophils that have been activated by means of preincubation with allergen express a high density of CD63 on their surface. The CD63-based flow assay involves incubation of heparinized whole blood with stimulation buffer, typically containing IL-3. Primed cells are then incubated in separate tubes with various concentrations of either purified recombinant or native allergens, crude allergen extracts, anti-IgE (positive control), or buffer (negative control for spontaneous expression). Basophils with surface receptor-bound IgE are then identified by means of gating flow cytometry with FITC-conjugated anti-human IgE in conjunction with phycoerythrin-labeled anti-CD63. Because CD63 is expressed normally on the inside of the vesicle membranes, where histamine is stored, the cross-linking of surface receptor-bound IgE fuses the vesicle with the plasma membrane. This allows CD63 to be expressed on the external surface of the cell in proportion to the amount of basophil activation. Quantitation of at least 500 basophils is performed by means of flow cytometry. The percentage of activated basophils is corrected for any spontaneous CD63 expression and evaluated as positive by using a variety of criteria, such as a stimulation index of greater than 2 (allergen induced/basal ratio).

In one study of 58 patients with a history of immediate-type reactions to β -lactam antibiotics and 30 nonallergic control subjects, the flow assay stimulation test (FAST) with CD63 as a marker for activation was evaluated by using minor determinant mixture, benzylpenicilloyl polylysine, penicillin, ampicillin amoxicillin, and cephalosporin as stimulating antigens.⁵⁷ Results were compared with drug-specific IgE levels, as measured in the Pharmacia

CAP System. The diagnostic sensitivity of the CD63 FAST was 50% relative to clinical history and improved to 66% by simultaneously using the drug-specific IgE antibody result. The CD63 FAST's diagnostic specificity was 93%. The authors concluded that the CD63 basophil activation test was helpful in supporting the diagnosis of IgE-mediated allergy to β -lactam drugs when it was used in conjunction with IgE antibody serology.

CD203c is a biomarker also known as neural cell-surface differentiation antigen, E-NIPP3 PD-1 β , 97A6, B10, and gp130rb13-6.⁵⁸ As a member of the ectonucleotide pyrophosphate phosphodiesterase family, it is expressed only on IgE-bearing basophils, mast cells, and their progenitors. In a manner analogous to CD63, CD203c is upregulated after activation of IgE-sensitized basophils with allergen or anti-IgE. The CD203c-based flow assay is analogous to the CD63-based assay described earlier. One advantage of using CD203c expression to identify basophil activation is its restriction to basophils in peripheral blood. With no concern for platelets and macrophages that may produce a false-positive CD63 result, there is no need for the use of an additional fluorescent anti-IgE reagent to gate the basophils.

In a study of patients allergic to Hymenoptera venom and healthy control subjects, basophils were analyzed for CD203c expression by means of flow cytometry after activation with anti-IgE or bee vespid venoms.⁵⁹ At 15 minutes after stimulation, 15-wasp sensitized patients had basophils that upregulated CD203c expression from 4.2- to 13.5-fold. Basophils from 6 patients with honeybee venom allergy upregulated CD203c by a mean of 8.3-fold. The CD203c-based flow assay was able to confirm the presence of venom-specific IgE antibody on basophils in 91% (20/22) of patients with venom allergy who had positive clinical histories and skin test responses, and one false-positive result was observed among 13 healthy control subjects with negative skin test responses.

One study has directly compared the relative diagnostic sensitivity of the 2 flow cytometric basophil activation protocols (IgE-CD63 and IgE-CD203c) after stimulation with natural rubber latex.⁶⁰ In this study processed blood from 27 patients with latex allergy and positive skin test responses and 12 subjects with negative skin test responses were evaluated. The authors observed a higher sensitivity with the CD203c protocol than the CD63 protocol (75% vs 50%, respectively), whereas the specificity for both protocols was 100%. Moreover, the observed magnitude of the basophil response was higher with CD203c than with CD63. The authors concluded that the fluorescent basophil activation simulation test was markedly improved by the use of the CD203c marker compared with CD63.

Diagnostic utility of basophil activation flow cytometric assays

A number of technical concerns related to the use of basophil assays in the diagnosis of allergic diseases continue to limit their application in clinical laboratories.⁶¹ Endotoxin-free whole blood must be shipped to the

laboratory, where it can be processed within 1 day by skilled technologists. The blood is then preincubated with stimulation buffer usually containing IL-3, which primes the basophils. Although IL-3 in the absence of other stimuli reportedly causes little or no histamine release or CD63 upregulation,^{62,63} false-positive mediator release or biomarker surface expression needs to be kept in mind as a possibility.⁶¹ The cells are then incubated with varying concentrations of crude allergen extract, recombinant allergen, or anti-IgE. Some crude extracts contain cytotoxins, and thus the stimulating allergens need to be prequalified for use with cells before they are actually used. Criteria for defining positive assay results vary with each stimulating allergen preparation because of variable potency. With CD63, there is the concern of false-positive results related to platelet adherence on activated basophils. Finally, when directly compared to puncture skin test results in multiple clinical studies with different allergen specificities, the diagnostic sensitivity and specificity of the basophil flow cytometric methods are found wanting. Therefore we anticipate continued use of these cell-based cytometric methods in research investigations of allergic disease but limited application to clinical diagnosis in the near future.

SUMMARY

Although the clinical history drives the diagnosis of allergic disease, by itself, it can be unreliable.⁶⁴ A number of *in vitro* assays of IgE in serum and on basophils have been developed to augment the clinical history and skin test results, especially when they are discordant. In this review serologic assays for IgE antibody are examined from their origin as qualitative assays to the present-day quantitative and automated third-generation methods. The second-generation serum-based IgE antibody assays have achieved a high degree of reproducibility and quantification. This has allowed probability curves to identify levels of specific IgE antibody above which selected food-induced and respiratory allergic disease is highly probable. The consequences have been an increased confidence in the diagnosis and a reduced need for provocation assays, especially in the diagnosis of food allergy, in which double-blind, placebo-controlled food challenges can be risky. At this time, clinicians should strive to obtain IgE antibody measurements from CLIA-certified clinical laboratories that perform, at the minimum, a second-generation quantitative IgE antibody assay. This focus on quantitative IgE antibody results will eventually lead to the phasing out of first-generation qualitative and semi-quantitative assays that report in nonquantitative signal units or arbitrary classes.

Although not discussed in this report, serum tryptase continues to be a useful biomarker for systemic anaphylaxis when an acute systemic reaction occurs. Assays for histamine and LTC₄ released from allergen-stimulated basophils have become more accurate, quantitative, and sensitive. The flow cytometric basophil activation assays

have recently provided a new technology in the identification of sensitized patients. However, because of their less than comparable diagnostic performance relative to skin tests and serology, continued constraints placed by the need for rapid processing of whole blood and the issues of optimizing allergen, criteria for defining positive results, and accounting for nonreleasers, the cell-based mediator release and flow cytometric assays will most likely remain useful research tools for the foreseeable future.

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