Clinical Commentary Review

Bringing the Next Generation of Food Allergy Diagnostics Into the Clinic

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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.

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List of Design Committee Members: Alexandra F. Santos, MD, PhD, Michael D. Kulis, PhD, and Hugh A. Sampson, MD (authors); Scott H. Sicherer, MD (editor)

Learning objectives:

1. To describe the principles of the basophil activation test (BAT) and mast cell activation test (MAT) and the determination of IgE to allergen epitopes.
2. To explain the diagnostic performance of the BAT and MAT and IgE to allergen epitopes in food allergy.
3. To describe the added value of the BAT and IgE to allergen epitopes to tests currently used in routine clinical practice.

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

Disclosure of Relevant Financial Relationships with Commercial Interests: A. F. Santos reports grants and personal fees from Medical Research Council (MR/M008517/1; MR/T032081/1); reports grants from Food Allergy Research and Education (FARE); Asthma UK and the National Institute for Health Research (NIHR) through the Biomedical Research Centre (BRC) award to Guy’s and St Thomas’ NHS Foundation Trust; and the Immune Tolerance Network/National Institute of Allergy and Infectious Diseases (NIH/NIAID); reports consultancy fees from Thermo Scientific; Nutricia, Infomed, Novartis, Buhlmann, Allergy Therapeutics, Novartis, Iggenix, Stallergenes; and reports research support from Buhlmann and Thermo Scientific through a collaboration agreement with King’s College London. M. Kulis reports funding through NIH/NIAID and the U.S. Department of Defense and consultancy fees from Ukko. H. A. Sampson receives funding to his institution for grants from the NIH/NIAID; reports consulting fees from DBV Technologies, S.A., N-Fold, LLC, and Siolta Therapeutic, Inc.; and reports stock options from DBV Technologies and N-Fold Therapeutics. The reviewer reported no relevant financial relationships.
Food allergy diagnosis has a massive impact on the lives of patients and their families. Despite recent developments with specific IgE to component allergens, a significant proportion of patients assessed for possible food allergy require oral food challenge to ensure an accurate diagnosis. More precise diagnostic methods are required to reduce the need for oral food challenges. Bead-based epitope assays and cellular tests, such as basophil activation and mast cell tests are the most novel and promising tests on the horizon. There is a pathway to pursue to enable their incorporation in clinical practice, including standardization, technical validation, clinical validation, external validation, overcoming practical and logistical issues, and regulatory approval. Valuable clinical application of these tests goes beyond diagnosis and includes risk assessment to identify allergic patients who are most sensitive and at risk for severe allergic reactions, and to define prognosis and assess clinical response to immunomodulatory treatments. © 2021 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/). (J Allergy Clin Immunol Pract 2022;10:1-9)

Key words: Basophil activation test; Mast cell activation test; Epitope mapping; Food allergy; Diagnosis

INTRODUCTION

Diagnosing food allergy has major implications for daily life. It may lead to dietary restrictions and constant risk assessment to minimize accidental exposure and allergic reactions. This in turn may cause anxiety of varying severity and limit activities in which individuals may engage, such as attending social events, eating in restaurants, and travelling. Therefore, overdiagnosis can have an unnecessary negative impact on patients’ lives. Conversely, failure to diagnose food allergy can place the patient at risk for reacting unexpectedly to food, with potential severe consequences. Thus, it is extremely important to get the diagnosis right.

Currently, food allergy is usually diagnosed based on a history of an acute allergic reaction that happens within 2 hours (usually minutes) of allergen exposure and evidence of allergen-specific IgE (sIgE) to the food in question. Allergen-specific IgE can be documented by skin prick test (SPT) or measurement of sIgE in the serum using allergen extracts or individual components, which can be either natural purified or recombinant proteins. Despite the improved accuracy conferred by sIgE to individual allergens for some foods, such as Ara h 2 from peanut and Cor a 9 and Cor a 14 from hazelnut, compared with IgE to allergen extracts, a significant proportion of patients who are assessed for possible food allergy require oral food challenge (OFC), which is currently the reference standard to diagnose food allergy. In fact, offering allergy tests such as SPT and sIgE testing ideally should come with access to OFC to minimize overdiagnosis of food allergy.

Oral food challenge is the current reference standard for diagnosis, assessment of severity, identification of the threshold dose of reactivity, confirmation of eligibility for treatment, and assessment of response to treatment. However, OFCs have limitations, require intensive resources, and can cause allergic reactions of varying severity. The need to undergo OFCs to confirm eligibility can leave patients and their families unsettled and anxious and may discourage allergic patients from considering specific treatments for food allergy. Therefore, improved biomarkers for food allergy are needed to close these gaps, provide an accurate diagnosis, and reduce the requirement for OFC.

The most novel and promising tests under way to support the diagnosis of food allergy, which are currently making the transition to clinical practice, are IgE to allergen peptides and cellular tests, such as the basophil activation test (BAT) and mast cell activation test (MAT). We will focus on these tests in the next sections. After describing these methods and their clinical utility, we will define a pathway to bring these new tests to the clinic and make them accessible to clinicians and patients with suspected food allergy.

THE NEXT GENERATION OF FOOD ALLERGY DIAGNOSTICS

Immunoglobulin E to epitopes

After the first in vitro assay capable of measuring sIgE to patient sera (radioallergosorbent test) in semiquantitative manner, a quantitative assay, ImmunoCAP (Pharmacia, Uppsala, Sweden), which was calibrated against the World Health Organization standard IgE, provided improved sensitivity and predictive value in diagnosing clinical allergy. The quantity of IgE specific to milk, egg, peanut, and fish was shown to correlate with the probability of reacting to the food compared with the double-blind placebo-controlled food challenge (DBPCFC), and 95% predictive value cutoffs were suggested. Component proteins comprising various food allergens (eg, milk casein, egg ovomucoid, peanut conglutins) eliminated many cross-reacting and clinically irrelevant allergen components and were more predictive of clinical reactivity than the whole food. Pursuing this reasoning further, researchers postulated that more precise IgE profiling (ie, evaluating epitope-specific antibodies found on allergenic component proteins) could improve diagnostic and prognostic methods by further eliminating irrelevant signals produced by cross-reactive and less specific antibodies. Early studies using SPOTS membrane technology suggested that IgE to sequential (linear) epitopes correlated most closely with persistent, systemic food-allergic reactions. Subsequent studies advanced to using microarray technology, and more recently to a high-throughput, highly reproducible bead-based epitope assay (BBEA).

As seen in Figure 1, BBEA is a multiplex assay capable of evaluating IgE and IgG4 to more than 90 peptide sequences representing various sequential epitopes. Advantages of BBEA include the microliter quantities of serum or plasma required to perform the assay, its technical ease of performance and
reproducibility, its greater degree of accuracy, and its potential to predict clinical outcomes. To date, most allergenic epitope profiling has focused on milk and peanut allergy (Table 1). Although this technology appears to hold great promise, it requires knowledge of the amino acid sequences of component protein allergenic epitopes comprising each individual food, large cohorts of well-characterized food-allergic patients to validate the assays, and extensive computational skills and machine learning to develop and validate diagnostic and prognostic algorithms for each food allergen.

**Basophil and mast cell activation tests**

More than the quantity of sIgE, the quality of sIgE is critical to determine the degree of mast cells and basophil activation, which is ultimately responsible for the allergic symptoms. Recently, Hemmings et al demonstrated that the proportion of IgE that is allergen-specific, the diversity of IgE response in terms of allergens that sIgE recognizes, the avidity for binding to the allergen, and sIgE levels all contribute to the proportion of mast cells and basophils that become activated in response to allergen stimulation. The beauty of cellular tests such as the BAT and the MAT is that they take all of the elements of sIgE into account when determining the likelihood that allergen exposure will lead to allergic reactions in an IgE-sensitized individual. The BAT uses whole blood whereas the MAT uses plasma or serum to sensitize mast cells that can be either a cell line or primary cells derived from peripheral blood or from tissue. These basophils or mast cells are then stimulated with allergen and analyzed by flow cytometry to detect the expression of activation markers before and after stimulation with allergens or controls (Figure 2).

Both allergen extracts and individual allergens can be used for cell stimulation. Their diagnostic performance differs depending on the allergen source. Generally, BAT has shown high sensitivity and specificity to diagnose peanut and other food allergies (Table 1). The MAT has shown similar specificity in the diagnosis of peanut allergy but lower sensitivity, because this is a more artificial system requiring antibodies in a fixed volume of plasma to bind to receptors on the surface of mast cells, and it depends on the density of such receptors on the cell surface. Because BAT and MAT require flow cytometry, with current methods it is not feasible to employ them as a screening test. Instead, they may be used as a confirmatory test, as a second step in the diagnostic workup in cases in which the first assessment with SPT or sIgE was inconclusive. Applied this way, the high specificity is the main added value to the SPT and sIgE, which have high sensitivity but relatively lower specificity. However, with more high-throughput methods, BAT, which also has high specificity, could be used more widely. Although BAT has high accuracy, approximately 10% to 15% of individuals with basophils who respond to non-IgE-mediated stimulants but...
not to IgE-mediated control or allergen (so-called nonresponders) have an uninterpretable BAT. For these people, MAT, which uses a passive sensitization system in which only the plasma comes from the patient, can provide a conclusive result.

**PATHWAY TO CLINICAL PRACTICE**

Different stages can be identified, from the development of a putative diagnostic test to its clinical implementation (Figure 3), such as standardization, technical validation, clinical validation, external validation, and overcoming practical and logistical issues, toward regulatory approval. We will address these in turn.

**Standardization**

Standardization of diagnostic tests allows for comparisons across time and laboratories and thus is vital to their development for clinical utility. For instance, in the BBEA, the length and sequence of peptides used on the beads; the manufacturer of the peptides; the type and size of beads; the equipment used to measure IgE binding; and the data analyses, which include machine learning, are some of aspects to consider. In the BAT and MAT assays, aspects to consider for standardization include allergen sources, cellular markers for identification and activation, and data analysis.

The allergen content of food extracts can vary widely depending on the source material and way in which the food was processed (eg, roasted vs raw peanut), as recently demonstrated for peanut. Source materials from extract providers with existing biological licenses, such as those used in SPT (eg, StallerGenes/Greer and ALK), which are provided in lyophilized form that can be readily resuspended, are useful for BAT and MAT. Purified allergens, or combinations of allergens, can also be used, but limited allergens are available. However, there are companies that offer standardized allergens for purchase (eg, Indoor Biotech, Charlottesville, Va). Another aspect of extract standardization is the concentration of proteins to be used in the assay. Typically, a log-fold dose range is used in BAT assays ranging from 10 μg/mL to 1 ng/mL. Because the BAT assay relies on peripheral blood, timing and transport of blood for testing must also be standardized. The BAT assays can be performed within 24 hours of blood collection; however, methods such as refrigeration of blood or fixing of samples before acquisition may extend this timing.

The BAT and MAT assays rely on flow cytometry to quantify activation. Therefore, the choice of cellular markers to target with antibody staining is important. Basophils can be identified by a variety of cellular markers including CD203c, FcεRI, IgE, CD123, and CCR3, and mast cells by c-KIT and FcεRI. Activation of basophils and mast cells is typically achieved by quantifying CD63 on the cellular surface. Basophil activation can also be measured by a change in the fluorescence of CD203c with allergen stimulation. Standardization of markers and antibodies to identify and activate these effector cells is critical and should balance the complexity of the flow cytometric acquisition of data with rigor to identify the correct cell population. Flow cytometry is routinely used in clinical laboratory settings and should be readily applicable for BAT and MAT assays. Data analysis also needs to be standardized. Ideally, this will move from manual gating strategies to more automated computer-assisted analysis.
Technical validation

It is important to validate BBEA, BAT, and MAT assays technically to transition these assays from research laboratories into clinical practice. For example, several operators will need to demonstrate the reproducibility of cellular activation on blood drawn from the same subject on the same day for the BAT assay in question. In addition, data generated from blood processed immediately after collection will need to be compared with blood processed several hours or possibly days later to reveal fluctuations in activation results and determine an acceptable window between blood collection and testing. Positive and negative controls must be included in the BAT assay, because high background activation could lead to false positives, and unresponsiveness to anti-IgE stimulation indicating a nonresponder in the assay could lead to false negatives. The MAT assay has an advantage in that cells are primed with plasma or serum; thus, the timing of the assay is not a major constraint as in the BAT. Ultimately, a coefficient of variation will need to be developed for replicates performed in BAT and MAT assays. These aspects of technical validation are being explored and are not seen to be major hurdles in implementing BAT and MAT for eventual clinical use. Similar aspects need to be covered for BBEA.

Clinical validation

Once a diagnostic test method has been optimized and standardized and has been shown to be consistent and reliable from a technical point of view, it needs to be clinically validated. That is, it needs to be compared with the reference standard for diagnosis to determine how results should be interpreted in relation to the clinical phenotype. In the case of food allergy, the reference standard for comparison ideally should be DBPCFC. Depending on the results obtained for a given test in individuals with and without allergy, cutoffs can be defined to determine when a test should be considered positive, and a non-dichotomous approach can also be adopted in which results can be associated with a certain probability of clinical food allergy.

Recently, a BBEA for the diagnosis of peanut allergy was validated for use in the United States using two independent cohorts from the CoFAR2 and POISED clinical trials. The peanut BBEA was found to diagnose 93% of subjects correctly, with 92% sensitivity, 94% specificity, 91% positive predictive value (PPV), and 95% negative predictive value (NPV). In other studies awaiting validation, epitope profiling has been shown to correlate with the severity of reactions to peanut and to distinguish milk-allergic children who will react to unprocessed milk, baked milk, or fermented milk products. For BAT to peanut, Santos et al identified the optimal cutoff that showed 97% overall accuracy, 98% sensitivity, 96% specificity, 95% PPV, and 98% NPV for peanut allergy. Similarly, for sesame and tree nuts, optimal cutoffs were identified and demonstrated an ability to reduce the number of OFCs, especially the number of positive OFCs in which patients would have experienced an allergic reaction of varying severity. Other studies looked at the utility of BAT to support the diagnosis of allergy to other foods.

Because different food allergies can be distinct in terms of the phenotype of patients (eg, in milk and egg allergies, an intermediate phenotype of patients tolerating baked milk or egg can be identified) and of the nature of the allergen that IgE recognizes, diagnostic tests can be interpreted differently and need to be validated for different food allergies. For example, 95% PPV cutoffs for SPT and sIgE to extracts and components are different for cow’s milk, egg, and peanut allergies. Other factors that can influence the cutoffs and the predictive value of a given test are the patient population, the geographic location, and the clinical setting. This explains the identification of different

![FIGURE 3. Test discovery and validation pathway.](image)
cutoffs for a given test, such as the sIgE determination whose method is standardized and widely available, in different patient populations. We anticipate that thresholds to confirm clinical reactivity using BBEA, BAT, and MAT will be different for varying foods.

External independent validation

As outlined in a publication by the Board on Health Care Services of the National Academy of Sciences, diagnostic tests must be rigorously developed and documented in accordance with national guidelines. Figure 3 shows the major steps in test development, which include test discovery, validation, and evaluation of clinical utility. In the discovery stage, a large, well-characterized cohort of specific food-allergic patients is divided into two sets: training and confirmation. The training set is used to establish the diagnostic standard or algorithm, which is then locked down before testing samples in the confirmation set. Unless the test performance of the confirmation set is in the range of the training set, it is likely that the diagnostic standard or algorithm has been overfit and will not be useful for other patient cohorts. Ideally, a second sample set from an independent cohort should be used, which will give a more realistic picture of how the diagnostic standard or algorithm will perform. The test can be further validated based on results of the analytic validation; it is then locked down before the final assessment of clinical utility. External independent validation has been completed for both the BBEA and the BAT to diagnose peanut allergy. For instance, the optimal cutoff defined for BAT in the primary peanut study was externally validated in an independent cohort with 100% specificity and 100% PPV. The same method for BAT was used in the Learning Early About Peanut allergy and associated studies, confirming the diagnostic value with the previously defined cutoff showing 99% specificity and 95% PPV.

Practical and logistical issues

The BAT assay requires peripheral blood, which needs to be processed soon after collection. This can raise practical issues with implementation in clinical practice. Another important consideration is transportation to the laboratory. Shipping at ambient temperature may not be ideal because fluctuations in environmental temperatures vary dramatically between seasons and geographic locations and may alter basophil reactivity. A possible solution is shipping under controlled temperatures using insulated boxes with elements that can regulate the temperature of the sample within a defined range, to avoid extreme temperature variations during transportation. An additional practical issue to consider is the use of anticoagulants. Heparinized blood is used in research settings for BAT assays; other tube types are also acceptable. However EDTA may inhibit basophil degranulation owing to calcium chelation, so calcium needs to be added before stimulation.

The MAT and BBEA use plasma or serum, which can be stored for a long time and tested when optimal conditions are achieved. Thus, logistical aspects to consider are, for instance, the storage space of samples for both MAT and BBEE and maintenance of the cell line in culture under appropriate conditions for MAT. Use of primary cells for passive sensitization assays adds practical challenges related to the standardization and
normalization of results given the variability of cellular reactivity between donors and over time.

Regulatory approval
In general, three pathways are available to obtain final US Food and Drug Administration (FDA) approval of a test, as depicted in Figure 4. Ideally, the clinical utility of a test should be derived from a prospective clinical trial. However, this is not always practical or feasible. In such cases, archived samples from prospective or retrospective studies may be used. If samples are derived from a prospective study, FDA consultation is typically obtained to determine whether an investigational device exemption is required (ie, for cases in which the outcome of the test will influence patient management). Depending on the study design and outcome, the FDA may grant approval of the in vitro test for general use or designate it as a laboratory developed test, which means the test is approved for use only in the single developing laboratory, before final approval. The FDA performs an in-depth review of the analytical and clinical validity of a test, whereas the Centers for Medicare and Medicaid Services (CMS) regulates all laboratory testing in humans in the United States through its Clinical Laboratory Improvement Amendments program. The CMS must also review and approve the performance characteristics of a test (eg, accuracy, reportable range, reference interval) before its availability for general clinical use.

RECOMMENDED CLINICAL APPROACH

Reality check
Obtaining final FDA and CMS approval in the United States for an early-stage diagnostic is like obtaining FDA approval for a new drug. Several phases of validation are required and can easily take 5 to 10 years. To date, there is one allergenic epitope-based diagnostic assay for peanut, VeriMAP Peanut (AllerGenis, Hatfield, Pa), which has 93% concordance with DBPCFCs and has achieved laboratory developed test status in the United States. Full FDA approval may occur within the next 1 to 2 years. Full FDA and CMS approval for similar epitope-based assays for milk and egg will likely take another 3 to 5 years, and full approval for other assays discussed in this review may take longer.

Indications
Because of improved accuracy in available diagnostic tests, the next generation of food allergy diagnostic tests should eliminate the need for most OFCs to confirm the diagnosis of food allergy. The BBEA has been independently validated to provide greater than 90% accuracy in diagnosing clinical peanut allergy. The BAT has demonstrated 97% diagnostic accuracy in a patient population recruited from a specialized allergy center covering a range of ages. In a selected group of patients who had an equivocal diagnosis following history, SPT and sIgE to peanut and to Ara h 2, BAT had 93% diagnostic accuracy. The MAT had about 88% accuracy in the same patient population.

Severity and threshold
Two studies using earlier microarray technology evaluated the correlation between allergenic-epitope diversity and the severity of clinical reaction to peanut. Immunoglobulin E diversity to sequential epitopes of Ara h 1, 2, and 3 correlated with reaction severity, as well as in vitro basophil cell degranulation. Studies are under way using the BBEA and machine learning to develop predictive algorithms that should predict clinical sensitivity more precisely (ie, eliciting dose and clinical severity).

Various studies showed that the proportion of activated basophils and mast cells in response to allergen is associated with the severity of allergic reactions and with a relatively low threshold of reactivity. Specifically, for peanut allergy, basophil activation greater than 48% identified subjects with severe and life-threatening reactions during peanut challenges with 100% sensitivity, 97% specificity, 41% PPV, and 100% NPV in the LEAP and associated studies. A BAT greater than 1.7% identified patients who reacted to 0.1 g or less of peanut protein during the OFC with 95% sensitivity, 91% specificity, 43% PPV, and 100% NPV in the same cohorts. An association among BAT, severity, and threshold was also established in earlier studies.

Nevertheless, the risk assessment of individual patients requires a holistic approach that considers the clinical context (eg, age, presence of asthma and other comorbidities) and psychosocial context (eg, anxiety, risk-taking behavior, access to emergency services) of patients and their families.

Prediction of response to treatment
Once a test has been validated for diagnosis, it may be used to follow response to treatment. The concept of monitoring treatment responses has become increasingly important. Because approval of the first food immunotherapy, Palforzia, occurred in 2020 and will likely result in more widespread use of oral immunotherapy, at least in the case of peanut allergy. Other biomarkers, such as sIgE, IgG4, and SPT, used to monitor oral immunotherapy have not been especially promising. The BBEA was shown to predict which milk-allergic children would achieve sustained unresponsiveness after oral immunotherapy, to predict infants at high risk who would go on to develop peanut allergy, and to predict the eliciting dose threshold of peanut-allergic children (unpublished).

Several examples of BAT assays after peanut, egg, and milk food-specific immunotherapy were published and are associated with desensitization. Similar suppression of basophil activation was demonstrated after peanut sublingual and epicutaneous immunotherapy, indicating that measuring BAT during immunotherapy may indicate a desensitized state. Considerably less is known about how the BAT can be used to determine sustained unresponsiveness; however, early reports are promising. For example, Patil et al described different trajectories of basophil response to Ara h 2 in patients treated with peanut oral immunotherapy over time, depending on their clinical response, either transient desensitization or sustained unresponsiveness. In that study, changes in basophil response as early as 3 months into treatment could predict the group in which patients would belong in terms of clinical response to treatment. Similar findings were reported by Chinthrajah et al. Based on selected baseline peptide-sIgE and IgG4 levels, Suarez-Farinas et al built predictive models to predict the likelihood of sustained unresponsiveness after milk oral immunotherapy, which was more predictive than models based on IgE to allergen components from cow’s milk. Similar findings were reported for peanut oral immunotherapy.

CONCLUSION
The BBEA, BAT, and MAT are the next generation of food allergy diagnostics, making the transition to clinical practice.
With the potential to enable more precise diagnosis and to reduce the need for OFC, it is important to gather the necessary evidence to pursue the steps toward regulatory approval of these new tests and their incorporation into clinical practice and guidelines for the benefit of patients with suspected food allergy.

REFERENCES


