

Advances in *in vitro* diagnostics in allergy, asthma, and immunology in 2012

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Laboratory tests play an increasing role in risk assessment, diagnostics, and disease monitoring. Great advances have been achieved lately, particularly in the field of clinical immunology and allergy. These include neonatal screening of immunodeficiencies and asthma biomarkers and investigation into the role of recombinant allergens in *in vitro* testing. The latter area has implications for the diagnostics of food allergy, pollen-induced allergies, asthma, and insect allergies. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: Immunodeficiencies, neonatal screening, recombinant allergens, food allergy, rhinitis, insect venom allergies

Laboratory tests play an increasing role as routine diagnostic procedures in both inpatient and outpatient services. The increasing knowledge about the mechanism and pathogenesis of diseases not only results in the development of novel therapeutic strategies but also in the discovery of novel biomarkers. With advent of personalized medicine, which is now on the horizon for allergy and asthma treatment, biomarkers are needed to guide therapeutic decisions. However, these biomarkers require careful clinical evaluation, and they need to be benchmarked against currently available gold standards.

The better understanding of disease mechanisms goes hand in hand with advancements in key technologies, which have been originally developed for research purposes and are now increasingly available for routine diagnostic procedures. Important examples include the following: flow cytometry, array- and chip-based diagnostic tools, mass spectrometry, and the development of recombinant tools. These techniques allow a broader and more detailed diagnostic assessment of the molecular and cellular

Abbreviations used

CCD: Carbohydrate determinant
CVID: Common variable immunodeficiency
FENO: Fraction of exhaled nitric oxide
GCR: Glucocorticoid receptor
SCID: Severe combined immunodeficiency
SLIT: Sublingual immunotherapy
TREC: T-cell receptor excision circle
WDEIA: Wheat-dependent, exercise-induced anaphylaxis

aspects of immune functions. Important examples represent recombinant allergens for the assessment of allergen-specific sensitization.

However, laboratory tests play an important role in more than just the establishment of clinical diagnoses. Increasingly, *in vitro* tests for risk assessment and stratification of patients are becoming available, leading to a personalized approach. Furthermore, diagnostic tests play an increasing role in assessing pharmacologic responsiveness or nonresponsiveness. Another important area is the use of laboratory tests in the identification of life-threatening severe diseases even before clinical onset of symptoms. This is the goal of neonatal screening, which has now also emerged in our field of clinical immunology.

In this article important advances in this area will be described that have been published in the *Journal of Allergy and Clinical Immunology* throughout the year 2012.

IMMUNODEFICIENCIES

Population-based neonatal screening has now arrived in the field of immunodeficiency diseases. Severe combined immunodeficiency (SCID) comprises a group of severe diseases affecting the immune system. Infants with SCID are healthy at birth but die of severe infections unless they receive hematopoietic stem cell transplantation, enzyme replacement therapy, or gene therapy. SCID is characterized by severe deficiencies in T- and B-cell function and also, in some types of the disease, natural killer cell function. This is a genetic disease, with mutations detected in more than 13 different genes thus far.¹ Among several methods that have been considered for SCID screening, quantitative assessment of T-cell receptor excision circles (TRECs) has been successfully validated.² TRECs are formed during recombination of the T-cell receptor α genes. During this process, DNA fragments are excised that are not further incorporated into the mature T-cell receptor locus, and they join at their ends to form DNA byproducts termed T-cell receptors. They are stable but do not replicate during cell division, resulting in a dilution of these byproducts throughout T-cell proliferation. Therefore TRECs

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Supported by the Deutsche Forschungsgemeinschaft (DFG; SFB/TR22), the German Lung Center (DZL), and the LOEWE Center "Universities Giessen and Marburg Lung Center" (UGMLC).

Disclosure of potential conflict of interest: H. Renz is a board member for DGAKI and the American Academy of Allergy, Asthma & Immunology; has consultancy arrangements with Allergopharma, ALK-Abelló, Bencard, Sterna Biologicals, Novartis, and Boehringer Ingelheim; has received one or more grants from or has one or more grants pending with DFG, BMBF, the European Union, Land Hessen, Stiftung Pathobiochemie, Ernst-Wendt-Stiftung, DAAD, and ALK-Abelló; and has received one or more payments for lecturing from or is on the speakers' bureau for Allergopharma, Novartis, Abbott, Med-update, and cmeAkademie.

Received for publication April 25, 2013; revised August 22, 2013; accepted for publication August 26, 2013.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2013.08.043>

are biomarkers for newly produced T cells, indicating a normal program of T-cell development.

Quantitative PCR assays have been developed to measure these byproducts. The DNA can be extracted from dried blood spots and collected on the well-known and widely used Guthrie card. This is already used for the screening of important metabolic defects. Starting with the US state of Wisconsin,^{3,4} almost 1 million TREC screening assays have been performed as of May 2011, detecting 14 cases of SCID, 6 cases of SCID variants, and 40 infants with various forms of T-cell lymphopenia.⁵ Recently, the first case of SCID identified in the course of such a pilot program in the state of Massachusetts has been published in this journal.⁶ Most states use homebrewed modifications of the TREC assay,⁷⁻¹⁰ with the exception of California, which is using a TREC assay under development by PerkinElmer (Waltham, Mass). The available data indicate that these assays fulfill the requirements for population-based screening because false-negative results, or the failure to identify true cases, is kept to an absolute minimum. This is to the expense of a small number of false-positive results, which require further follow-up. Thus far, the tests have proved very sensitive but not completely specific.²

Further follow-up of positive screening results include qualitative and quantitative assessment of lymphocytes and lymphocyte/T-cell subsets. However, the age-adjusted reference range in the newborn period has to be taken into consideration.¹¹ As an alternative for the DNA-based TREC assay, a method based on tandem mass spectrometry has been also proposed.¹² This assay was developed to detect adenosine deaminase deficiency, which is one cause of SCID (an autosomal recessive form), leading to the accumulation of toxic metabolites in the purine salvage pathway. These metabolites are detected by using this method. The assay was validated in a pilot population-based screening program in Tuscany, Italy, and compiled 4 dried blood-spot samples from patients with genetically confirmed adenosine deaminase-deficient SCID and more than 12 thousand samples from healthy newborns. Recently, several guidelines for population-based⁸ and patient-centered¹³ have been proposed.

The most common symptomatic primary immunodeficiency is common variable immunodeficiency (CVID). The disease is most frequently diagnosed in adults age 20 to 40 years, and there still is a 6- to 8-year delay in establishing the diagnosis in many patients. CVID is associated with a number of chronic complications, including autoimmunity, chronic lung disease, inflammatory bowel disease, systemic granulomatosis, and lymphoid hyperplasia malignancy. A genetic cause of CVID is in most cases unknown, and polymorphisms have been described in a number of genes. A recent article highlights the laboratory diagnosis in these patients.¹⁴ Reduced levels of total IgG, IgA, and/or IgM and a demonstrated deficiency in specific antibody production are essential criteria. The total IgG level is generally less than 400 mg/dL, and if IgG levels decrease to less than 200 mg/dL, initiation of immunoglobulin replacement therapy is strongly recommended.

The qualitative assessment of antibody function is of great importance in the diagnostic work-up of primary immunodeficiencies. Diagnostic vaccination and measurements of the specific antibody responses against such standardized antigens represents a well-established routine procedure. A recently published document by the American Academy of Allergy, Asthma & Immunology Working Group of the Basic Clinical Immunology Interest Section¹⁵ provides a state-of-the-art

overview on this procedure and interpretation of the results. Despite a frequent use of diagnostic vaccination, the authors conclude that there is a need for further studies to firmly establish a normal range of antibody titers in response to vaccine antigens, particularly to such antigens with which there is a variable response. Furthermore, they point out that in the end, the clinical status of the patient dictates the therapeutic intervention and not solely the test result.

Administration of standardized immunoglobulin preparations represents an important therapeutic approach to various antibody deficiency diseases. As a relatively rare but nevertheless severe side effect, anaphylactic reactions to immunoglobulin infusions have been reported, and they were attributed in patients with undetectable IgA to the presence of IgG or IgE antibodies directed against IgA. Reports of reactions to immunoglobulin products in IgA-deficient patients have been recently reviewed.¹⁶ On the basis of a thorough examination of these cases, the authors conclude that IgG therapy should never be withheld from an IgA-deficient patient solely because of concerns about the risk of rare adverse events. Furthermore, they conclude that there are insufficient data to warrant a general recommendation for screening for anti-IgA antibodies in these patients because the predictive value of a positive result is still not established. Subcutaneous infusions appear to be tolerated by patients with anti-IgA antibodies.

A recent review and evaluation of a patient with hyper-IgM syndrome¹⁷ pointed to the immunologic phenotype of this condition. For a number of different reasons, IgM B cells have a defect in class-switch recombination, which could be due to defects in cell-cell interaction, intrinsic defects in B-cell development, or DNA repair mechanism. Importantly, the authors point out that patients present with low IgG, IgA, and IgE levels together with either normal or increased IgM levels. Because IgM levels are not necessarily high, they claim the term hyper-IgM as a misnomer.

ALLERGY AND ASTHMA

Asthma biomarkers

The area of personalized and stratified therapeutic approaches has now also arrived in the field of asthma therapy. A small but considerable number of asthmatic patients have poorly controlled disease with standard therapy. Several recently published clinical trials illustrate that such patients might benefit from treatment with biological agents targeting cytokines of the T_H2 immunobiology (eg, IL-5 and IL-13) if they present with an immunomolecular phenotype of high T_H2 responses.^{18,19} Therefore there is the need for the development of blood tests to accurately predict treatment responses in such patients.²⁰

Periostin represents a biomolecule that has recently received major attention in this regard. It has been shown that periostin promotes eosinophilic inflammation in response to T_H2 cytokines.²¹ However, upregulation of periostin is not restricted to this type of inflammation but is rather considered a tissue response to injury and stress. Periostin is expressed in the epithelium of the respiratory tract and gut. Its levels are increased in patients with certain cancers, fibrosis, pulmonary hypertension, and dermatitis.²²⁻²⁵

A set of biomarkers was recently evaluated in 67 asthmatic patients who remained symptomatic despite maximal inhaled corticosteroid treatment.²⁶ The aim of the study was to identify systemic biomarkers of eosinophilic airway inflammation, which

was assessed based on sputum and results of bronchoscopy. Compared with fraction of exhaled nitric oxide (FENO) values, blood eosinophilia, and IgE levels, blood periostin levels achieve the highest sensitivity and specificity in the receiver operating characteristic analysis, with an area under the curve of 84%. Even in a logistic regression model of biomarkers versus eosinophil status, serum periostin reached the highest level of significance at a *P* value of .007. However, further studies are required to establish this marker as a systemic parameter of airway eosinophilia in asthmatic patients and its usefulness in selecting stratified therapeutic approaches in these patients.

Corticosteroid resistance represents an increasingly recognized problem in the treatment of asthmatic patients.²⁷⁻³⁰ A recently published study sought to investigate PBMC biomarkers as predictors of clinical responsiveness.³¹ The authors investigated steroid responsiveness in a group of patients with uncontrolled asthma and correlated responsiveness with a set of biomarkers. As a result, they found that the responsive subgroup of patients had a higher baseline level of mitogen-induced kinase phosphate 1, IL-8, and glucocorticoid receptor (GCR)- β mRNA. Furthermore, they had a lower GCR- α /GCR- β mRNA ratio and were less responsive to the suppression of TNF- α and IL-8 by dexamethasone. These data represent an important starting point for the search for reliable biomarkers to identify such patients. Further larger and independent clinical studies are required for validation.

Also, biomarkers are playing an increasingly important role as outcome parameters in clinical studies. The National Institutes of Health and federal agencies of the United States convened an expert panel to propose useful biomarkers in this regard. In an elegant systematic approach the panel published its recommendations recently.³² Although 10 parameters were identified by the panel, only 1 was recommended as a core asthma outcome: multiallergen screening to define atopy. Blood and sputum eosinophil counts, FENO values, urinary leukotriene levels, and total and allergen-specific IgE antibody titers were recommended as supplemental measures. The panel recommends that bio-samples should be stored in biobanks and standard procedures should be developed to harmonize sample collection and analysis.

Farmer's lung disease

Serologic diagnosis of farmer's lung disease, which is the most frequent occupational hypersensitivity pneumonitis, still represents a great challenge. In a recent elegant report,³³ investigators used sera from such patients to screen for antigenic proteins from ascospores (*Aspergillus* species). They then produced a panel of recombinant antigens and identified 2 proteins that were effective in differentiating between patients with farmer's lung disease and exposed but healthy farmers by using an ELISA test (area under the curve, 0.89 and 0.87, respectively). This approach can serve as a starting point to improve serology for such hypersensitivity diseases.

A major area in which recombinant allergens are diagnostically applied is in the field of type I hypersensitivity responses. Major highlights from the last year follow.

Food allergens

The identification of peanut allergy based on component-resolved diagnostics has provided new insights into clinically

relevant sensitization (Table I). The major peanut allergens Ara h 1, Ara h 2, and Ara h 3 are the proteins considered primarily responsible for sensitization to peanut. Ara h 2 is resistant to heat and gastric denaturation and plays a major role in diagnostics. A recent study³⁴ performed open oral challenges with peanut in 144 children sensitized to Ara h 8 but not to the other major peanut components. They found that isolated Ara h 8 sensitization indicates tolerance to peanuts in almost all cases. They also cautioned that sensitization against thus far unidentified components in peanut might cause symptoms in rare cases. On the basis of a study by Dang et al,³⁵ the accuracy of peanut allergy diagnostics can be increased by applying a combinatorial approach of testing specific IgE antibodies against whole peanut, followed by Ara h 2. This study was driven by the question of whether more accurate diagnostics could minimize the need for oral food challenges, which still represent the gold standard. In their study with 200 children, half of them with peanut allergy, the number of oral food challenges required to accurately diagnose the disease could be reduced by almost two thirds by using this novel stepwise approach. Sublingual immunotherapy (SLIT) is currently under clinical investigation as a novel treatment for peanut allergy. In a recent study³⁶ the usefulness of peanut-specific IgA levels in saliva was evaluated during SLIT. Using a panel of 10 SLIT-treated and 7 placebo-treated subjects, the authors found that salivary anti-peanut IgA might serve as a potential biomarker to follow throughout therapy and could be useful in determining the efficacy of therapy to monitor the progress of treatment. Further studies are needed to validate this biomarker.

Egg allergy is among the most common food allergies worldwide. A study performed in Japan³⁷ aimed to determine the positive predictive value of IgE antibodies against the 2 major egg allergy components, Gal d 1 (ovomucoid) and Gal d 2 (ovalbumin), for predicting the results of oral food challenges with boiled egg white in children without previous (known) egg exposure. They found that measurement of IgE antibodies against Gal d 1, which is considered the dominant allergen in boiled eggs or heated egg products, yields better results than measurement of IgE antibodies against Gal d 2. The role of IgG₄ antibodies against the same allergens was evaluated in a similar patient population. One hundred seven children with egg allergy were challenged to baked egg, and the outcomes of the challenges were related to the level of specific IgE/IgG₄ antibody titers against Gal d 1 and Gal d 2.³⁸ The authors developed a mathematic model that includes the interaction between IgE and IgG₄ to Gal d 1 and Gal d 2 and found that their model accurately predicts reactivity to baked egg. However, further studies are needed to validate this approach.

Food-dependent, exercise induced anaphylaxis

Wheat-dependent, exercise-induced anaphylaxis (WDEIA) is a special form of wheat allergy induced by the combination of wheat ingestion (bred, pizzas, and pasta) and physical exercise. WDEIA is the best characterized form of food-dependent, exercise-induced anaphylaxis. Establishing the diagnosis is still problematic because provocation test results are only positive in about 60% of patients and in only 30% of patients with commercially available wheat extract. Recently, the use of recombinant wheat allergens has been found to be increasingly helpful in the diagnosis of WDEIA. In particular, omega-5-gliadin

TABLE I. Key advances in the field of allergy diagnostics using recombinant allergens in 2012

Allergen	Component(s)	Comment
Peanut	Ara h 1, Ara h 2, Ara h 3	Primarily responsible for sensitization
	Ara h 8	Isolated positivity: marker of tolerance ³⁴
	Ara h 2	Plus native peanut: reduction of required OFCs
Egg	Gal d 1	Ovomucoid = heat resistant
	Gal d 2	Ovalbumin ³⁷ Mathematic model: IgG/IgG ₄ against Gal d 1 + Gal d 2 for diagnosis of egg allergy ³⁸
Soy	Gly m 4	PR-10 family; good predictor ⁴⁵
Wheat	Tri a 19	Omega-5-Gliadin; WDEIA (high sensitivity and specificity) ³⁹⁻⁴³
Bee venom	Api m 1	No binding of CCD-reactive IgE, but patients with low-level IgE against natural allergen might be missed ^{52,53}
Wasp venom	Ves v 5, Ves v 1	Combined use of both components greatly enhances sensitivity ⁵³⁻⁵⁵

OFC, Oral food challenge; PR-10, pathogenesis-related class 10 protein.

(Tri a 19) is of importance in this regard, increasing sensitivity to approximately 80% with high specificity. Tri a 19 is a storage protein and has been identified as the major allergen in patients with WDEIA.³⁹⁻⁴³ Still, there remains a diagnostic window. This could be closed in the future by using additional recombinant proteins, such as the high-molecular-weight glutenin (Tri a 26), α/β -gliadin (Tri a 21), and γ -gliadin. However, further studies are necessary. The role of the basophil degranulation test (CD203c expression) was investigated in another study.⁴⁴ The authors found that hydrolyzed wheat protein enhanced CD203c expression in a dose-dependent manner in patients with hydrolyzed wheat protein anaphylaxis, whereas omega-5-gliadin enhanced CD203c expression in patients with conventional anaphylaxis to wheat. However, larger studies are needed to confirm these markers as predictors to differentiate between these 2 subtypes of WDEIA.

Soybean is another important food allergen, particularly during childhood. A recent study⁴⁵ confirmed that sensitization to Gly m 4 is also a reasonable predictor in adult patients with soybean allergy. However, double-blind, placebo-controlled challenges were not performed in this study. Gly m 4 is a member of the pathogenesis-related class 10 protein family, which has high homology to Bet v 1, the major allergen of birch pollen.

Pollen allergens

A cross-sectional observational study was performed in 200 children with allergic rhinitis, asthma, or both to assess the molecular sensitization profile to *Phleum pratense*, which is one of the most important grass pollen allergens in southern Europe. Among the 176 children with IgE sensitization to the whole allergen extract, 39 profiles of sensitization to 8 allergenic molecules have been identified. Consistent with other studies, Phl p 1 and Phl p 5 are the most important allergenic proteins, followed by Phl p 2, Phl p 4, and Phl p 6. IgE responses to

Phl p 11 and Phl p 12 and especially Phl p 7 are less frequent. This study indicates that the sensitization profile to *P. pratense* is highly heterogeneous, and the authors further conclude that the molecular design of specific immunotherapy preparations should consider this high heterogeneity of sensitization patterns. It will be of great interest to analyze the longitudinal development of such sensitization profiles in these patients.⁴⁶

Asthma

The role and effect of specific sensitizations on asthma development, exacerbation, and severity have been extensively examined throughout the last year. Participants from the European Community Respiratory Health Survey II were tested with the commercially available microarray chip technology ISAC against 103 allergen molecules.⁴⁷ The investigators found that simultaneous sensitization to perennial, pollen, and food allergens involves the highest risk of asthma (odds ratio, 18.3). In comparison with increased FENO values, the presence of IgE antibodies against food allergens is independently associated with increases in the risk of asthma in such patients. Furthermore, FENO values, bronchial hyperresponsiveness, and risk of asthma increase with multiple sensitizations to different allergen groups.

IgE antibodies against *Staphylococcus aureus* enterotoxins have been implied as important risk factors for nasal polyps. A recent study examined whether IgE to such superantigens might be relevant to disease severity in adult asthmatic patients. The authors measured specific IgE antibodies in serum against enterotoxins and grass pollen and house dust mite allergens in a cohort of 152 patients with nonsevere asthma, 166 patients with severe asthma, and 69 control subjects. They found that 21% of patients with severe asthma and positive enterotoxin IgE titers were considered nonatopic. Further analysis revealed that IgE antibodies against the enterotoxins but not IgE against inhalant allergens are risk factors for asthma severity. Whether IgE antibodies against enterotoxins are also involved in the pathogenesis of asthma severity and exacerbation remains to be examined.⁴⁸

Rhinovirus infection represents an important trigger for asthma exacerbation. A study performed in Costa Rica⁴⁹ examined the prevalence of rhinovirus infections in relation to the atopic status of children. Atopic status was analyzed by measuring levels of allergen-specific IgE antibodies, particularly those against house dust mite. They found high titers of IgE antibodies to dust mites in many infants, and these IgE antibodies increased the risk for acute wheezing provoked by rhinovirus infections. Therefore the presence of IgE antibodies against house dust mite could represent an additional important risk factor. Whether this can be also applied to other geographic regions needs to be examined.

Hymenoptera

Several articles investigated and discussed the usefulness of recombinant allergens in improving the diagnostics of insect venom allergies (Table I). Double positivity to bee and wasp venom and cross-reactivity with carbohydrate determinants (CCDs) still represent important clinical problems. In one article⁵⁰ a combinational approach of using recombinant insect venom allergen to measure IgE antibody titers together with the basophil activation test has been proposed to improve diagnostic specificity in patients with a doubtful diagnosis. Oral CCD exposure is very common, and recently, it has been shown that

alcohol drinkers (heavy drinkers) with no allergic symptoms might have high CCD IgE antibody titers, which can trigger a positive basophil activation test result not only to CCDs but also to CCD-bearing allergens, such as bromelain, pollen, and insect venoms. This possible interference in this particular patient group should be kept in mind.⁵¹ The expression system used to produce recombinant venom allergens in the ImmunoCAP System (Phadia, Uppsala, Sweden) is based on the insect cell line Sf9, which is defective in the synthesis of glycans, representing major determinants of CCD reactivity. In a brief study comparing natural and recombinant Api m 1, the investigators found comparable results in CCD-negative patients with bee venom allergy, except for a minority of patients displaying low levels of IgE to natural Api m 1, which might be missed by using recombinant Api m 1. However, the authors conclude that the use of recombinant Api m 1 is of greater benefit because it does not bind CCD-reactive IgE antibodies and therefore detects true sensitization to bee venom.⁵² However, this conclusion is not unopposed,⁵³ and further studies are needed. In the case of wasp allergy, the combined use of recombinant Ves v 5 and Ves v 1 greatly enhanced the sensitivity of the test, but still approximately 8% of patients with true wasp allergy remain undetected.⁵³ Therefore diagnostics with natural allergen should be the first line of laboratory evaluation.^{54,55}

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