Molecular Allergen-Specific IgE Assays as a Complement to Allergen Extract–Based Sensitization Assessment

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List of Design Committee Members: Rob C. Aalberse, PhD, and Joost A. Aalberse, MD

Activity Objectives

Learning objectives:

1. To understand the current and future possibilities and limitations of component-resolved diagnostic (CRD) allergy tests.
2. To decide how to implement CRD in daily practice.
3. To understand the diagnostic implications of the cross-reactivity among allergens.

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Molecular allergen–based component-resolved diagnostic IgE antibody tests have emerged in the form of singleplex assays and multiplex arrays. They use both native and recombinant allergen molecules, sometimes in combination with each other, to supplement allergen extract–based IgE antibody analyses. The total number of available allergenic molecules has reached a diagnostically useful level; however, more molecules are needed to cover all the clinically important allergen specificities. Thus, for the foreseeable future, molecular allergen-specific IgE analyses will remain a supplement for initial allergen extract–based IgE antibody analyses in the diagnostic workup of the allergic patient. As a spin-off, it will enable manufacturers to improve the quality of extracts for *in vitro* testing. The 2 most exciting diagnostic developments linked to component-resolved diagnostic tests are the possibility to increase diagnostic sensitivity by the inclusion of allergens that are underrepresented in the current extracts and *in vitro* assays and to increase the diagnostic specificity by taking the information on allergen cross-reactivity into account. Particularly the latter application is still under development. This requires additional studies on the clinical relevance of serological cross-reactivity. © **2015 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2015;3:863-9)**

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Molecular allergology is a discipline that encompasses individual biomolecules that are involved in IgE antibody responses or react with IgE antibodies. This article focuses on the

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Abbreviations used
CRD- component-resolved diagnostics
LTP- lipid transfer protein

measurement in serum (or other body fluids) of IgE antibodies to "molecularly defined" allergens. It is potentially a source of confusion that an "allergen" can refer to an allergen source as well as to allergenic molecular structures. If there is risk of ambiguity, we will specify the term: "allergenic source" or "molecular allergen." In the strict sense, "molecular allergen" is a pleonasm because all allergens are molecules. In his original description in 1991, Valenta et al[1] suggested the term "component-resolved diagnostics" (CRD). More recently, the World Allergy Association has coined the term "molecular based allergy diagnostics,"[2] in which a test measuring IgE antibody to a single component is referred to as measuring IgE to a single molecule. Because "component" seems more appropriate than "molecule," we use CRD in the following text, because it more clearly describes a diagnostic test that targets single types of allergenic molecules.

Not all allergens that are in extracts have been currently defined at the molecular level. Other allergens have been well characterized but have not yet been produced with the quality that is required for CRD tests. These additional allergens are needed to determine whether a particular subset of components will be sufficient to identify sensitization patterns in all patients with symptoms upon exposure to the allergen source material in question. In addition, CRD-derived information can be used to improve the quality of allergen mixtures including allergen extracts, which is essential for optimization of the adequacy of recombinant allergen panels.

ALLERGEN SOURCE COMPLEXITY

Most allergenic sources contain more than a single type of molecular allergen. In the 1970s, extracts of grass, ragweed, birch, cat, dust mite, and cod fish were thought to have a single major allergen, and perhaps a number of minor allergens. It is now clear that the average number of known allergen types per source material is usually more than 5. This includes neither the subtle variations between isoallergens nor those alterations that may occur after the biosynthesis of the allergen. The more that is known about the allergenic molecules and their structure, the more similarity and variability becomes evident both within and between source materials. For instance, the allergenic molecules in pollens from temperate grasses are structurally similar, whereas the major allergens are different for the grasses, trees, and mites. However, we have a number of allergologically similar allergens that cross species barriers. These are often referred to as pan-allergens. Well-known examples are evolutionary conserved muscle proteins (particularly tropomyosin) from diverse invertebrates, such as shrimp, mites, mollusks, and silverfish, as well as plant profilins, which are highly conserved between grasses and trees (monocots and dicots, respectively). As will be discussed in more detail below, this high similarity results in widespread cross-reactivity. Other allergens have a more restricted cross-reactivity spectrum. IgE antibodies to the major birch allergen Bet v 1 often cross-react with related proteins in other dicots, but not with monocots. IgE antibodies reacting to mouse urinary protein often cross-react with other rodent urinary lipocalins, but not to beta-lactoglobulin, a lipocalin in cow's milk.

It makes sense to cluster molecular allergens into allergen families, but it can also be confusing. Two allergens are called cross-reactive if they can compete for the same IgE antibody. If the amino acid sequences of 2 allergens are more than 70% identical, these allergens are often cross-reactive, whereas cross-reactivity becomes rare if sequence identity is less than 50%. However, even at much lower sequence identities (as low as 15%), 2 allergens may be evolutionary related. This is a cause of confusion because these allergens are not cross-reactive but are still considered to belong to the same protein family. As mentioned above, many allergens belong to the lipocalin protein family, but they often have little or no cross-reactivity. Two major dog allergens Can f 1 and Can f 2 are lipocalins, but they have only 19% sequence identity and no cross-reactivity. From a practical allergological point of view, the degree of cross-reactivity among allergens is more relevant than their evolutionary family relationship. Quantitative information on cross-reactivity is still hard to find and may be dependent on the geographical area. Sequence identities of allergens from 6 families are presented in Figure 1 as a surrogate marker for cross-reactivity. The muscle protein tropomyosin is the most conserved protein among these 6 families, with more than 40% sequence identity between invertebrate allergens and the human protein. Because human tropomyosin usually induces immune tolerance also to epitopes that are conserved between mammals and invertebrates, the potential contribution to cross-reactivity of amino acids that are identical between mite and shrimp is lower than the simple comparison between mite and shrimp indicates (Figure 2). This makes the prediction of cross-reactivity among tropomyosins less reliable than for allergens without a human homologue. As indicated in Figure 2, the number of identical amino acids decreases from 227 to 84 if a correction is made for identical amino acids in human tropomyosin (which are likely to contribute to tolerance rather than to cross-reactivity). This is a simplistic approach to cross-reactivity prediction because no distinction is made between surface-exposed and buried amino acids and so forth. For a more in-depth discussion on the relation between sequence identity, cross-reactivity, and clinical reactivity, see Aalberse et al[3] and Aalberse.[4]

TWO CAUSES OF POLYREACTIVITY THAT NEED TO BE DISTINGUISHED ARE MULTISENSITIZATION AND CROSS-REACTIVITY

"Polyreactivity" is a term used to indicate that a serum contains IgE antibodies to many allergens. This phenomenon has 2 nonexclusive explanations: multisensitization and cross-reactivity. Multisensitization refers to the induction of unrelated IgE antibody responses, whereas cross-reactivity refers to a single instance of IgE antibody induction, resulting in antibodies with reactivity to several (cross-reactive) allergens. In this section, it will be explained why and how to distinguish these 2 explanations.

With the introduction of CRD, the term "polyreactivity" has acquired a new dimension. It now not only encompasses reactivity to several allergenic source materials but also the condition in which a serum has IgE antibody that binds to several allergen components from a single source material. Reactivity to multiple allergens from closely related grasses is not counted as polyreactivity because the structures of these allergens are considered

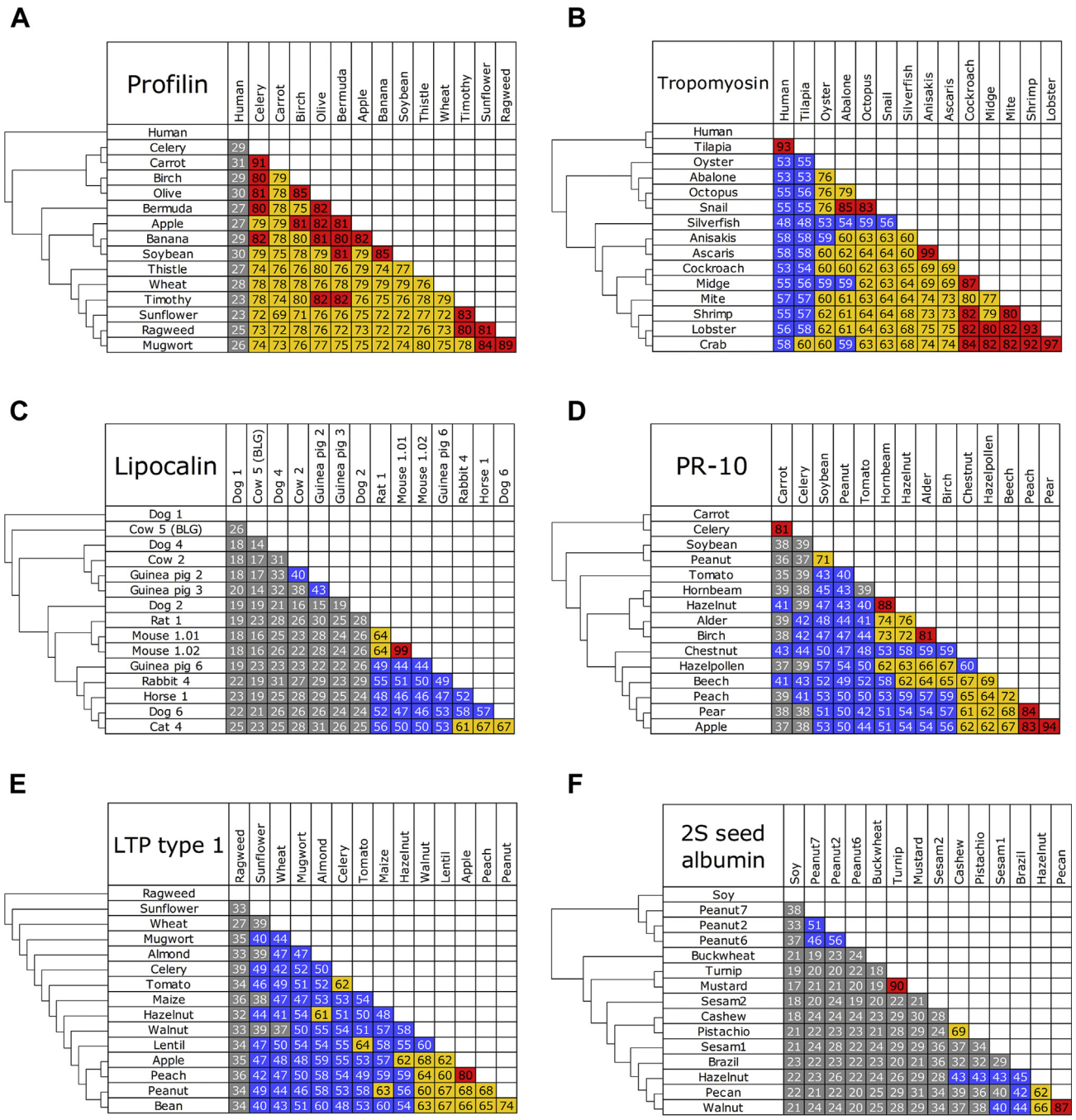

FIGURE 1. Sequence homologies among allergens grouped by protein family (A-F). The numbers indicate the % amino acid sequence identity. The likelihood of cross-reactivity decreases from red (>80% sequence identity) via orange and blue to grey (<40% sequence identity, unlikely to be cross-reactive). Among the lipcalins, Dog 1 stands for Can f 1, Dog 4, for Can f 4, etc. Among the 2S seed albumins (F), peanut 2 stands for Ara h 2, etc. LTP = Lipid transfer protein; PR-10 = pathogenesis-related protein 10.

to be virtually identical. The major birch allergen (Bet v 1) and its structural homologue in apple (Mal d 1), however, are considered to be different. Allergens that are not too different may be cross-reactive. Cross-reactivity and its assessment have been discussed in more detail elsewhere.[3,4] This term is often applicable to the birch-apple situation. Another example is the cross-reactivity between shrimp and house dust mite due to ingesting shrimp tropomyosin, which often results in IgE antibodies that cross-react with mite tropomyosin.[5] If a serum is positive both to shrimp and to mites, this possibly reflects a single type of IgE antibody that reacts with tropomyosins from both sources (Figure 3). In contrast, if a serum is positive for lactoglobulin from cow's milk and to Can f 1, a major allergen in dog saliva, this is almost certainly a case of multisensitization, even if these 2 latter proteins belong to the same allergen family (lipocalins).

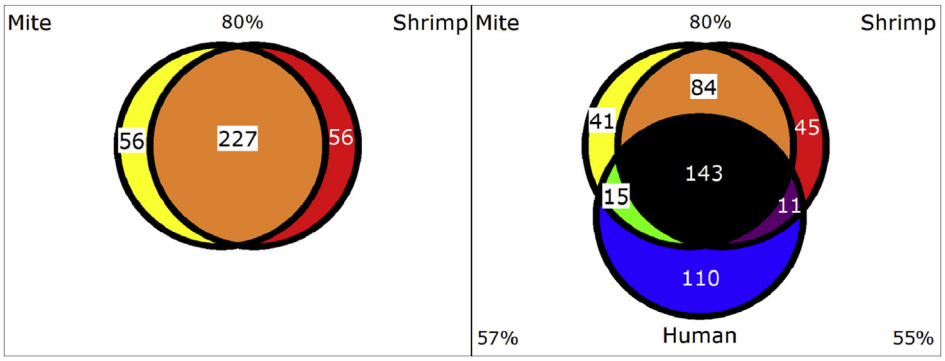

FIGURE 2. Illustration of the potential influence of sequence identity between human tropomyosin and invertebrate tropomyosins on the cross-reactivity among invertebrate tropomyosins. The numbers in the Venn diagrams indicate the number of identical amino acids among mite, shrimp, and human tropomyosins. The numbers outside the Venn diagrams indicate the sequence identity as percentage.

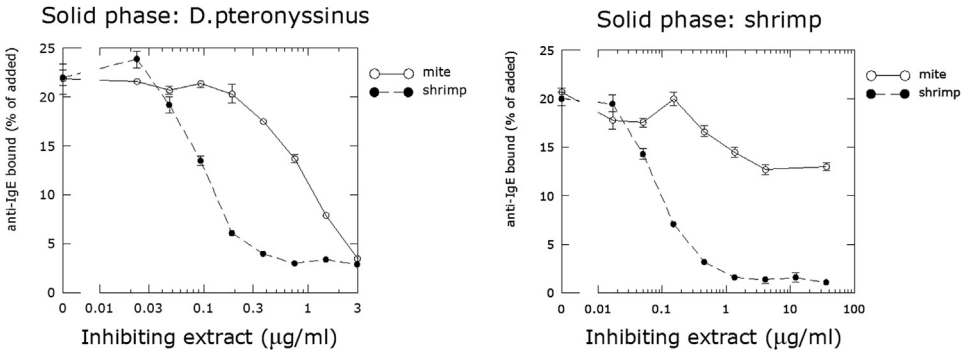

FIGURE 3. Illustration of the use of a quantitative crossed inhibition test. It shows that an allergen in shrimp extract (shrimp tropomyosin, as was shown by using an antitropomyosin mAb) fully inhibits IgE binding to mite tropomyosin, Der p 10. The mite extract only partially inhibits IgE binding to shrimp extract. Because the mite extract does not inhibit all IgE antibodies to shrimp tropomyosin, whereas the shrimp extract inhibits all IgE antibodies to mite tropomyosin, shrimp is more likely to be the sensitizer than mite. From Witteman et al.[5]

These 2 situations (cross-reactivity and multisensitization) can be discerned by using IgE inhibition tests. The basic principle is that IgE antibodies that are reactive to one allergen are pretreated with another allergen (suspected to be cross-reactive) to neutralize allergen reactivity to this latter allergen. Subsequently, a test is performed to measure residual IgE reactivity (if any) to the first allergen. In the apple-birch example, pretreatment of serum with the birch allergen will remove all reactivity to apple, whereas pretreatment with the apple allergen will remove only some of the birch reactivity. The most likely explanation of these results is that the birch allergen is the sensitizing allergen. Exposure to birch triggered the induction of these IgE antibodies, and some of these antibodies happen to react with the homologous allergen in apple. In the other example, neither pretreatment with lactoglobulin nor pretreatment with the dog allergen will affect IgE reactivity to the other allergen. This indicates multisensitization.

As we will discuss in more detail later, it is clinically relevant to distinguish multisensitization from cross-reactivity. Unfortunately, these IgE inhibition tests are considered research tools and they are not used as part of the standard IgE antibody testing. Thus, a next-best approach is to rely on statistical information, using the percentage of sequence identity of 2 allergens (Figure 1), and population-based data on multisensitization and cross-reactivity in the relevant geographical context. Data on the individual's environmental exposure may also be helpful in deciding between multisensitization and cross-reactivity.

HOW ARE "CRD" TESTS PERFORMED?

IgE antibodies to individual allergen components have been detected using various approaches. One method has been to purify an allergenic component by physicochemical means or by affinity chromatography using allergen-specific mAbs. A second approach was to incorporate an allergen-specific mAb as an immobilized capture reagent in an IgE antibody assay.[5] A third approach used immobilized synthetic peptides that contained some of the allergen's binding epitopes. Most of the initial data on IgE antibody profiles to allergenic components was obtained using a combination of electrophoresis and immune precipitation in agarose gel (Crossed Radio-Immuno Electrophoresis[6]) or blotting onto nitrocellulose.[7] All these historic approaches remain useful, but each has distinct limitations.

The development of the current much more versatile approaches required the large-scale availability of allergens obtained

by recombinant DNA technologies, usually referred to as "recombinant allergens." IgE antibodies to these allergenic components can be tested in a multiplex system (eg, microarrays and fluorescent multicolored beads) in which a serum sample is tested for reactivity to many individual allergens. Alternatively, a panel of traditional singleplex assays is used, in which a separate serum sample is needed for each allergen. The multiplex platforms are analytically not as robust as singleplex tests. An obvious difference is the amount of serum needed for testing a large number of allergens. A less obvious, but more marked difference is the amount of allergen used per data point. The 2 extremes are the ImmunoCAP singleplex assay (>1000 ng/test) and the multiplex ISAC microarray (<0.001 ng/test), more than a millionfold difference. Low allergen levels increase the risk of interference by non-IgE antibodies. The effect of interference by non-IgE antibodies (largely IgG_4-blocking antibodies) is as expected: In the ImmunoCAP, interference by blocking antibodies becomes noticeable only if the total antibody level (IgE + IgG) exceeds approximately 100 ng/test or 2500 ng/mL sample. For the microarray, the effect depends on the antibody ratio IgE/(IgE + IgG), as expected and experimentally confirmed by Lupinek et al.[8] This implies that the test result, and thus the lower limit of detection, is inversely proportional to the allergen-specific IgG level in the serum even at relatively low IgG levels.

Allergen component– versus extract–based serological tests for *in vivo* and *in vitro* diagnosis of allergic sensitization

For the foreseeable future, allergen extracts will remain indispensable reagents for *in vivo* and *in vitro* assays that identify patient sensitization. However, regulatory pressure in both Europe and North America may negatively affect the availability of extracts for *in vivo* testing, which will have repercussions for their *in vitro* use.[9,10] The availability of recombinant allergens is the single most critical technical hurdle to their application in the diagnostic tests that are used to assess a patient's sensitization profile. The currently available panel of allergenic components is incomplete. This is especially true for the complex allergen source materials such as cockroach and many molds and foods. Even for foods that are considered well covered such as peanut,[11,12] hazelnut,[13] and shrimp,[14] tests with all currently available allergenic components do not reach 95% diagnostic sensitivity. Moreover, natural allergens are needed to verify the quality (potency and immune reactivity) of available recombinant allergenic proteins. Thus, extract-based serological assays for IgE antibody will remain indispensable, even if they are imperfect.

Clinically irrelevant IgE antibody results that are analytically positive in asymptomatic subjects are commonly encountered in population surveys. For instance, in some geographical areas, more than half the test results showing IgE to peanut extract are from individuals who are peanut-tolerant. Conversely, insufficient analytical sensitivity in comparison to the results of *in vivo* provocation tests can occur as a result of insufficient levels of allergens in an extract. For instance, Cor a 1.04, the pathogenesis-related protein 10 (PR-10) family allergen in hazelnut, which is different from Cor a 1.01, the PR-10 allergen in hazel pollen (Figure 1), was found to be underrepresented in hazelnut extracts used in the preparation of allergosorbents.[15] A similar underrepresentation has been described for the homologous allergens in peanut (Ara h 8).[12] Spiking hazelnut extracts used in serological IgE antibody assays with Cor a 1 caused a marked increase in IgE antihazelnut positivity, with a concomitant increased detection of clinically irrelevant IgE antibodies.[16] Although it is likely that in many cases IgE to these PR-10 proteins is induced via airborne exposure to pollen-derived PR-10 rather than by ingestion of food-derived PR-10, food-derived PR-10 proteins may substantially contribute to food-induced allergic reactions in some patients. If so, it makes sense to include food-derived PR-10 protein(s) in the allergen test protocol, either by spiking the extract or by performing a separate PR-10 test. Methods to deal with the increased rate of clinically irrelevant but analytically true positive test results will be discussed subsequently. Increasing the number of allergy tests may be too costly (in serum and analytical costs) if done by a singleplex procedure. This does not necessarily force an investigator to switch to a microarray. A hybrid solution would be to combine similar allergens (such as PR-10 proteins) into a single "singleplex" test.

Claims that the diagnostic sensitivity of a test based on a combination of recombinant allergens is as good as that of tests based on optimal allergen extracts need to be carefully scrutinized.[12] Because conventional extracts used to qualify subjects used in these studies may be deficient in relevant allergens, some informative patients may have been excluded from testing because of selection bias. This invalidates the equivalent sensitivity claim in the assay that uses combinations of allergen components. Current studies comparing allergenic extracts and components indicate that more clinical experience is needed with patients who have been characterized using more comprehensive extracts before the decision can be made that we can do without extracts in serological IgE antibody assays.[12]

CLINICAL APPLICATIONS OF COMPONENT-BASED IgE ANTIBODY TESTS

Allergenic molecules are gradually becoming integrated into daily diagnostic routines as a support to extract-based analyses. It is likely that in the not-too-distant future (10 years?), the most important extract-based tests will gradually start to be replaced by tests based on cocktails of recombinant allergens. Elsewhere, we stressed the need for thorough validation protocols before such a replacement is considered because it is likely to be a 1-way street with no return possible to extract use.[12]

How this integration can be optimally accomplished is a subject of debate. As for the evaluation of the performance of these diagnostic tests, it will need to incorporate information on the pretest probability that exposure to allergen(s) is the cause of the reported patient symptoms. In published studies on the value of diagnostic tests, this information can usually be calculated from the results of provocation tests, but is nevertheless not always easily extracted from the article. In daily practice, a clinician can often make a rough estimate of the pretest likelihood from a patient's history in combination with historical data from similarly diseased patients from the same geographic area. For some patients, the pretest probability that an allergen exposure will induce an allergic symptom is close to 100% (or to 0%). In this situation, an IgE antibody test is not generally needed to confirm the diagnosis. However, even in these clearcut cases, tests for sensitization to assess cross-reactivity may be useful by providing a ranked list of potential pathogenic (cross-reacting) allergens. For such applications, a multiplex assay such as a microarray

assay may be most well suited. In the more typical diagnostic situation with a pretest probability between 25% and 75%, additional tests are warranted, but extract-based tests will often suffice.

In more complicated cases, component-based IgE tests can provide additional information that will strengthen the diagnosis in various ways.

A false-negative IgE antibody result in a patient with a positive allergen challenge can occur because of a deficiency in a relevant allergen extract used as a reagent in the assay. This may be prevented by performing an additional test with the specific allergen molecule that is known to be missing, or, as mentioned earlier, by spiking the extract. Examples of this include the oleosins and lipid transfer proteins (LTPs) in nuts and peanuts or Can f 5, the prostate-derived allergen from dog.[14] A better solution might be to request that the suppliers of the extracts revise their extraction protocol to include low-abundance allergens in the extract.

Sometimes, cross-reactivity can enhance a test's ability to detect a particular IgE antibody specificity. In diagnostic tests for shellfish allergy, for instance, it is usually sufficient to use one species to detect sensitization to another species even if it is taxonomically only distantly related. The allergens causing most of this cross-reactivity are muscle proteins such as tropomyosin and arginine kinase. It has been found that some patients without IgE to tropomyosin yet have polyreactivity to many types of shellfish.[17] This finding suggests that other broadly cross-reactive allergens exist. It may be worthwhile to identify these hypothetical additional cross-reactive allergens and use these, possibly in combination with tropomyosin, to create a "generic shellfish" test, that is, a single test that can efficiently detect sensitization to a wide variety of shellfish.[18] Cross-reactivity is an important cause of clinically misleading positive IgE antibody test results. IgE antibody to peanut extracts can be positive in birch-sensitized patients due to IgE anti−Bet v 1 that cross-reacts with its peanut homologue, Ara h 8. This is particularly encountered as a consequence of the use of a wide allergen screen in which a peanut test is performed without indication, or in epidemiological surveys. In theory, it is possible to perform an IgE inhibition test by adding soluble birch pollen extract to the serum and repeating the peanut specific IgE analysis. However, this is never done in practice because of the cost and time of this additional analysis. A more practical but equally costly solution is to perform a peanut Ara h 1, 2, 3, 6, and 8 specific IgE panel using singleplex assay analysis. The diagnostic accuracy of the single-component tests that are currently available is not yet sufficient, but this may change. It is possible to combine several components into a single test. Such a combination of specific allergens may improve clinical sensitivity in a single test. For cross-reactive allergens as well as for more specific allergens, IgE antibody responses to some allergenic molecules are considered indicators of an increased risk for more severe allergic reactions. LTPs in fruits, nuts, peanuts, and cereals are examples. Severe reactions to fruits, particularly peach, are much more likely to be caused by LTP than by PR-10 allergens due to their higher stability to heat and digestion. However, caution should be exercised because high levels of IgE antibodies to PR-10 proteins can be detected in the serum of patients with severe food allergy symptoms[12] and high levels to LTP-specific IgE are sometimes found in food-tolerant subjects.[19] It is thus important to keep in mind that the clinical relevance of cross-reactive sensitization is not as clearcut as is sometimes assumed. Studies combining quantitative cross-reactivity measurements at the component level and comprehensive clinical data are needed to establish an evidence-based diagnostic protocol.

Polyreactivity (particularly if caused by multisensitization) may be relevant as a marker for a more severely allergic phenotype. This could be a reflection of higher and more frequent and/or prolonged allergen exposure and the subsequent persistence of allergen-driven inflammation at more diverse sites of exposure. Multiplex tests are convenient ways to establish polyreactivity at the molecular level. However, the most relevant type of polyreactivity is at the allergenic source level. Such polyreactivity can be established by traditional extract testing.

One potential use of multiplex tests is in the identification of the offending source material (if any) in patients with "idiopathic" allergy.[20] The large number of allergens in the multiplex test (for microarrays currently 100-200) may suggest an almost full coverage of the allergen repertoire. Unsurprisingly, most allergens in a microarray are derived from the standard extract repertoire. It is therefore doubtful that a microarray test would be capable of detecting allergen sources that would escape detection by standard extract-based allergy tests. However, with the identification of new minor allergens used either for spiking the conventional extract or in the microarray, more patients will probably be identified.

In conclusion, component-resolved IgE antibody assays have great potential for improving the detection of a patient's sensitization profile. Their use can improve the analytical and clinical sensitivity of IgE antibody assays by replacing underrepresented allergens in current extracts. Improved assay specificity can be achieved by detecting IgE antibodies to source-specific allergens. It will in the future hopefully enable the identification of the most important allergen source materials from a ranking of the reactivity to a panel of cross-reacting allergens. If patient-tailored immunotherapy via components becomes a reality, CRD is an important, if not essential, tool to defining appropriate allergen specificities for treatment. The Holy Grail in allergy diagnosis is, however, to increase specificity while maintaining a diagnostic sensitivity level of better than 95% in a population with a 50% pretest probability of having allergic disease. Although more than 95% diagnostic specificity is an unrealistic target for serological IgE antibody analyses with more than 95% sensitivity, the additional information provided by molecular allergen−based IgE antibody tests may promote better identification of sensitized subjects with a low risk of clinical reactivity that can safely be subjected to an open allergen challenge in a nonclinical setting. Additional studies on the clinical relevance of serological cross-reactivity are among the requirements to reach this goal.

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